Cell-Specific Growth Inhibition of Human Cervical Cancer Cell by Recombinant Adenovirus p53 in vitro and in vivo

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Purpose: Despite the significance of the p53 adenoviral vector in cancer gene therapy, an advanced strategy for the development of preferential tumor cell-specific delivery and the long-term persistent gene expression control of p53 are required. In this study, the time-course expression patterns of p53 and E6, on cervical cancer cells, were investigated to obtain a molecular level understanding of the cell-dependent tumor growth suppression effects of a recombinant adenovirus expressing p53, both in vitro and in vivo.

Materials and Methods: The expressions of p53 and E6 in CaSki, SiHa, HeLa, HeLaS3, C33A and HT3 cervical cancer cell lines were examined. After infection with AdCMV-p53, the cell growth inhibition was studied via cell count, MTT and Neutral red assays. After transfecting the AdCMV-p53 and AdCMVLacZ into the cancer cells-xenografted nude mice, the anti-tumor effects were investigated for one month.

Results: The p53 protein levels were more notably expressed in the CaSki and HeLa than in the SiHa and HeLaS3.

On day 6, the p53 was only detected in the HeLaS3. In contrast, the p53 expression was highly maintained in the C33A and HT3. The E6 mRNA levels gradually decreased in only the CaSki and HeLa. The growth suppression effects also showed cell-dependent patterns, which were consistent with the reciprocal expression patterns of p53 and E6. After transfection of the AdCMVp53, into the CaSki- and SiHa-xenografted nude mice, the tumor size was remarkably decreased in the SiHa cells as compared to that in the AdCMVLacZ transfected mice, indicating cell-specific growth inhibition patterns.

Conclusion: The adenovirus-mediated p53 gene transfection was very effective both in vitro and in vivo. Also, the anti-tumor effects were accomplished via the differential role of p53-specific apoptotic cell death, which was dependent on the cervical cancer cell line. (Cancer Research and Treatment 2003;35:181-190)

Key Words: Cervix neoplasm, Gene therapy, p53 gene

INTRODUCTION

The tumor suppressor gene, p53, has been noted as an essential component in the suppression of tumor cell growth (1), which codes for a transcription factor consisting of 393 amino acids of 53 kD. The p53 protein induces growth arrest, or apoptosis, and takes part in the regulation of the cell cycle as a transactivator, which acts to negatively regulate cell division by controlling a set of genes required for this process (2). Most cancer development frequently comes from p53 gene mutations. p53 mutation occurs in more than 50% of cancer cells. In normal cells, DNA damage induces the expression of p53, which promotes G1 arrest or apoptosis. Dysfunction of p53 causes uncontrolled cell growth that enhances a tumor (3). Thus, as a basic gene therapy for cancer, the replacement of the altered gene causing the carcinoma has been suggested. However, the correction of genes is still only possible in vitro. HPV 16 and 18 have been identified in over 90% of all cervical cancer (4). In patients with cervical cancer, there are few cases where the p53 gene is inactivated due to allelic loss or point mutation. Overall, point mutation of the p53 gene is known to be present in about 1 ~ 6% of human carcinomas (5). Generally, after a high-risk HPV infection, the E6 and E7 oncoproteins are consistently expressed, and essential for the immortalization and transformation of human squamous epithelial cells (6). The E6 and E7 proteins form complexes with p53 and Rb, respectively, inhibiting the activities of the proteins in
cell cycle regulatory systems (7). These selective degradations of the tumor suppressor proteins are strategically very important in gene therapy, as the inactivation of p53 and Rb are essential for the induction of a cervical carcinoma (8). It is well known that cervical cancer cell lines infected with HPV express normal pRB, but only low-levels of the wild type p53. Trials on the suppression of cancer cell growth, via the restoration of the p53 mutation, have progressed considerably. The p53-dependent apoptosis has been regarded as a useful strategy for treating human cancer (9).

The adenoviral vector has been used to transfer specific genes into cells (10), generating an overwhelming amount of data and literature, but as yet remains far from ideal in clinical cervical cancer therapy, as the effects of adenoviral vectors in human than in animal models may be different. Also, p53 can induce multiple independently regulated apoptotic pathways, depending on the physiological circumstances and cell type, but the exact molecular mechanism leading to p53-mediated apoptosis is still unclear. It is now increasingly uncertain whether there are wide variations in the transduction efficiencies and tumorigenicity inhibitory effects among different cell types (11).

In this study, a recombinant AdCMVp53 was constructed, and the effect of the p53 adenoviral vector, on cervical cancer cells, was evaluated to investigate the molecular pathway of the cell-dependent tumor suppression effects both in vitro and in vivo. Also, the molecular basis of the tumor-specific effects, concerning either p53-dependent or independent apoptosis, was studied using the E6 oncogene expression. This report shows that p53 delivery results in a more differential growth inhibition pattern in cervical cancer cells both in vitro and in vivo. This inhibition appears to be mediated by p53-dependent apoptosis and cell cycle arrest, which was dependent on the cervical cancer cell line. Also, the tumor-specific growth suppression effects showed the differential regulations of the expressions of the E6 oncogene and p53.

**MATERIALS AND METHODS**

1) Cell lines and culture conditions

Four HPV-infected human cervical cancer (HPV 16-positive cells, CaSki and SiHa cells; and HPV 18-positive cells, HeLa and HeLaS3 cells) and HPV negative C33A and HT3 cell lines, with a mutation on the p53 gene were used. The cell lines were obtained from the cell line bank at Seoul National University’s Cancer Research Center. The cells were routinely propagated in monolayer cultures in DMEM (GIBCO-BRL, Paisley, Scotland), supplemented with 5% heat-inactivated fetal bovine serum, 0.37% sodium bicarbonate, 30 mM HEPES and penicillin/streptomycin. The cells were cultured in a 5% CO₂ incubator at 37°C. Unless otherwise specified, all chemicals were purchased from Aldrich and Sigma Chemical.

2) Recombinant adenoviral vector and titration of AdCMVp53 viral stock

A recombinant adenoviral vector, AdCMVp53, containing the human wild-type p53 gene, was constructed as follows: A 1.7 kb wild-type p53 gene, with the HCMV promoter sequence, was cloned into the EcoRV and XbaI sites of pΔE1spIB, and then co-transfected with the adenovirus plasmid, pBHGE3, into a 293 cell, and cultured in complete media for about 12 days. The p53 recombinant adenoviruses were isolated from a single plaque, expanded into a 293 cell and purified with a double CsCl gradient using ultracentrifugation (25,000 rpm, 4°C and 2 hrs). The final infectious titers were determined as 10⁸ plaque-forming units (pfu) per ml. The AdCMVp53 was provided by Dr. Jae-Ho Lee of Seoul National University, Seoul Korea. To determine the transfection efficiency, the AdCMVp53 was used as previously described (3).

The viral titer was determined, by the plaque assay method, in the 293 cells, as previously described (10). Briefly, 293 cells (10⁵ cells/well) were inoculated into a 12 well plate and cultured for 24 hr. When 90% confluency was achieved, the cells were infected with the recombinant adenovirus. And then, the cell plates were shaken 6 times for 1 h and then 1 ml of cDMEM was added. The cell cytopathic effect (CPE) was observed after 12, 24, 26 and 48 hr using a converted microscope.

3) Western blotting hybridization

The cells extract underwent electrophoresis for 2 h with SDS-PAGE at 10 mA, and the blotting performed for 1 h and 30 min with a Hybond-ECL membrane (Amersham, Upppsala, Sweden) at 100 volts. The blotted membrane was blocked with 5% skimmed milk and reacted with a primary antibody, mouse anti-human p53 DO7 (Novocastra, Newcastle, UK), and a secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (Novocastra, Newcastle, UK). For each step, the membrane was washed three times with TBST (Tris buffered saline with 0.1% Tween-20, pH 8.0). Protein bands were visualized using an ECL Kit, according to the manufacturer’s protocol (Amersham, Arlington Heights, IL).

4) Cell growth suppression and IC₅₀ assay

Cervical cancer cell lines were first inoculated from the 12 well plates at 5×10⁶~5×10⁷ cells/wells, cultured, overnight, in DMEM containing 10% PBS, washed with 1 ml of DPBS, infected with recombinant adenoviruses at dilutions of 31.25, 62.5, 125, 250 and 500 MOI (multiplicity of infection) and cultured for 1 h. The number of cells was then measured every day under microscopy for the growth suppression analysis. The cell number was an average of triplicate measurements.

5) Transfection efficiency

In order to confirm the transfected LacZ gene expression and the transfer efficiency, the AdCMVp53 was infected into the 293, and the six cervical cancer cell lines using the same method as for the infection of p53 adenovirus to the cells. The wells, washed with PBS, was fixed with 0.5% glutaraldehyde solution for 10 min. 2 ml of 5×buffer, containing 0.05% sodium deoxycholate and 0.1% NP-40 and 10 mM MgCl₂ were mixed with 0.1 ml 100×X-Gal (Promega, Madison, WI), 0.1 ml 100×K₃Fe(CN)₆ and 0.1 ml 100×K₄Fe(CN)₆ and 7.7 ml of DPBS (Gibco), to prepare X-Gal (Promega, Madison, WI) staining solution. 1 ml of this solution was placed into each cell culture, and incubates at 37°C for 1~2 h. The number of the stained cells was counted with a microscope (200 and 400 magnification) to determine the transfection efficiency.
6) MTT and neutral red assays

Tumor cells were seeded at 10^3 cells/well (0.1 ml) in 96 well flat-bottomed plates and incubated for 24 hr at 37°C. For the MTT assay, 20 μl of 5×MTT was added to each cell culture well and cultured for 3 h. 200 μl of DMSO was added to the culture, shaken for 10 min and the absorbance measured with an ELISA-reader at 570 nm.

0.2 ml of neutral red solution, pre-warmed to 37°C, was added to the cell culture wells and incubated for 3 h at 37°C. After discarding the solution, 0.2 ml of rinse solution (37% formaldehyde, 1% CaCl2 (w/v)) was added, react for 1~2 min, and then discarded. 0.2 ml of solubilization solution (1% acetic acid in 50% EtOH) was added to each well and shaken for 15 min. The absorbance was measured at 540 nm with an ELISA reader.

7) 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. The cell viability was confirmed by trypan blue dye exclusion.

8) RT-PCR

Total RNA was isolated from infected cells using Tri-reagent (Gibco), which were used as templates. After the pure mRNA had been separated it was quantified to an equal weight of 100 ng using an UV spectrophotometer and Et-Br staining plate assay. Reverse transcription was performed at 22°C for 10 min, and then at 42°C for 20 min, using 1.0μg of RNA per reaction. PCR was performed on the HPV 16 positive cell lines, CaSki and SiHa, employing 35 cycles at 94°C for 1 min, at 57°C for 1 min and at 72°C for 1 min using the HPV 16 primers (forward: 5’-GAGAATGCAATGTTTCAGGAC-3’, reverse: 5’-CCACCGACCCCCATATATATTAGG-3’) to obtain the PCR products. PCR was performed on the HPV 18 positive cell lines, HeLa and HeLaS3, employing 35 cycles at 94°C for 1 min, at 57°C for 1 min and at 72°C for 1 min using the HPV 18 E6 primers (forward: 5’-GATGTAAGAAACACCCACA-3’, reverse: 5’-CATAGAAAGCTCCAGCGAAT-3’). PCR of the p53 gene was also performed using the p53 primers (forward: 5’-GAAGCAGTCACAGCAATGC-3’ and reverse: 5’-GAGTCTTCCAGTGAATG-3’), which were used to confirm the recombinant AdCMVp53 virus. The PCR was performed with 35 cycles at 95°C for 30 sec, at 56°C for 30 sec and at 72°C for 1 min. The specific primers, forward: 5’-TGACGGGTTCAACCCACACTGTGCCCATCTA-3’, reverse: 5’-CTAGAAGCATTGGCCTGGACGGTGGAG-3’, were used for the control gene β-actin. The amplification reaction involved denaturation at 94°C for 30 sec, annealing at 72°C for 30 sec and 72°C for 45 sec for 20 cycles.

9) Inhibition of tumor growth

Cancer cells (10^3 cells/mouse), in 0.1 ml PBS, were injected into 6-week-old female mice. Seven mice were used for each cancer cell injection. Six days later, a solution containing 5×10^5 pfus of AdCMVp53, AdCMVLacZ, or PBS was injected into the area where tumor cells had generated. The tumor formation and size were evaluated once every two days for 4 weeks, by measuring two perpendicular diameters with calipers, and tumor size calculated based on average dimensions. The tumors were resected on the days indicated, and frozen to -70°C until required for analysis. The total RNA and total protein were extracted with Trizol, as described in the manufacturer’s protocol, for RT-PCR and western analyses.

10) β-galactosidase immunohistochemistry

After injection of AdCMVLacZ (5×10^9 pfus/mouse), the mice were sacrificed, and tumor tissue samples obtained according to the regular order. Sections of tissues were fixed in 4% formaldehyde at 4°C for 3 hr, washed three times in PBS and stained with X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) solution at 37°C for 4h, resulting in a blue color in the β-galactosidase-expressing tissues. After washing in PBS for 3 hr, the samples were immersed in several concentrations of alcohol, acetone, xylene and paraffin, and then fixed on a tray. The tissue samples were mounted on slides in 20μm sections.

11) p53 immunohistochemistry

The tissue samples were fixed with 4% formalin for over 24 hr, and washed. The samples were immersed in several concentrations of alcohol, acetone, xylene and paraffin, and then fixed on a tray. The tissue samples were mounted on slides in 20μm sections. Tissue samples were deparaffinized in histoclear and xylene, and then dehydrated by rinsing twice with 100% alcohol. The slides were blocked with acidic alcohol and incubated with the primary antibody of p53 for 1 hr at room temperature. After rinsing with PBS, the slides were incubated with the secondary antibody for 30 min at room temperature, immersed in PBS, reacted with detection buffer for 15 min at 45°C. AEC (3-amino-9-ethylcarbazole) was used to detect the antigen-antibody complex. The slides were visualized using microscope, washed with distilled water and counter-stained with hematoxylin.

12) Statistical analysis

The statistical analyses were performed using the paired Student’s t test and ANOVA. The values between the different groups were compared, with a p values 0.05 considered statistically significant.

RESULTS

1) IC₅₀ and transfection efficiency of AdCMV

After incubating for 48 hr at 37°C, the cytopathic effect (CPE) in the cells was observed using a converted microscope. The cytotoxicity of the adenovirus vector, at 0~500 MOIs, was determined in order to exclude the cytopathic effect of the adenovirus for each cell line. The AdCMVLacZ showed no cytotoxicity in the cells at 0~100 MOI, but above 500 MOI, the cytotoxicity was observed in all the tested cells. The IC₅₀ for each cervical cell line was as follows; CaSki (68.5 MOI), SiHa (43.5 MOI), HeLa (31 MOI), HeLaS3 (42 MOI), C33A (21 MOI) and HT3 (62 MOI) (Fig. 1). To further evaluate the transfection efficiencies in the cell lines, the cells were infected with different amounts of AdCMVLacZ, ranging...
Fig. 1. Growth-inhibitory effects of adenovirus p53 delivery in various cervical cancer cell lines at different MOI(s). Cells ($10^6$ 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, the cells were cultured for 2 days and then trypsinized for counting under a microscope. The mean values of the cell counts from triplicate measurements are plotted: Open bar (AdCMVlacZ); closed bar (AdCMVp53).

from 1 to 100 MOI, for 1 day. The results showed that infection of cells at 100 MOI resulted in 100% X-gal-staining of every cell line. Furthermore, infection of the CaSkI, SiHa and HeLaS3 cells, at an MOI of 50, showed 60 to 75% transfection efficiencies. Taken together, these data suggest that recombinant AdCMV vectors can infect different cell lines in a similar fashion.

2) Cell growth inhibition by AdCMVp53

To evaluate the differential effect of the expression of the
exogenous wild-type p53 on the growth of the cervical cancer cell lines, the cancer cell lines were transfected with AdCMV-p53 for 2 days, with increasing MOIs. As shown in Fig. 1, infection with an increasing number of adenoviruses resulted in the inhibition of the cell growth in a MOI-dependent manner. In particular, complete inhibition of the cell growth was observed at 125 MOI in both the CaSki and SiHa cells. However, complete inhibition of the cell growth was detected at 62.5 MOI in the HeLa and HeLaS3 cells. In contrast, at these MOI, no suppression of the cell growth was observed when the

![Graphs showing growth inhibition effects of adenovirus p53 delivery in various cervical cancer cell lines.](image)

**Fig. 2.** Growth-inhibitory effects of adenovirus p53 delivery in various cervical cancer 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, the cells were cultured for various periods and then trypsinized for counting under a microscope. The mean values of the cell counts from triplicate measurements are plotted. Mock-infected, AdCMVLacZ-infected, AdCMVp53-infected.
cells were infected with the recombinant adenovirus expressing β-gal as a negative control, suggesting that inhibition of the cell growth was mediated solely by the expression of the exogenous p53.

The degree of cell growth inhibition was measured via cell counts and MTT and Neutral red assays with increasing times, and showed a significant increase in the cell growth inhibition over time. In contrast, the infection with AdCMVLaCZ showed an increased cell growth in a manner similar to the negative control group. As shown in Fig. 2, the inhibitions of the cell growth, indicated from the cell counts, were 97.4, 81.6, 95%, 85%, 94 and 74%, for the CaSkI, SiHa, HeLa, HeLaS3, C33A and HT3 cell lines, on day 6, respectively. Where as the MTT assay showed 81, 47, 78.5, 51, 94 and 76%, for the CaSkI, SiHa, HeLa, HeLaS3, C33A and HT3 cell lines, on day 6, respectively. Those for the neutral red assay showed 57, 29, 37, 43, 60 and 65%, for the CaSkI, SiHa, HeLa, HeLaS3, C33A and HT3 cell lines, on day 6, respectively. Note that the same suppression level trend was shown in the CaSkI and HeLa, and in the SiHa and HeLaS3 cells.

To determine whether the AdCMV53 vector overexpresses the p53 protein in each cell line, western blot analyses were performed after the transfection of the AdCMVp53 into each cell line (Fig. 3). The results showed that the expression of the exogenous p53 protein at a high level in the HPV positive cells on days 2 and 4, but not on day 6. Conversely, that in the C33A and HT3 cells maintained a high level of expression. Note that the same trend in the levels of p53 expression was shown in the CaSkI and HeLa, and in the SiHa and HeLaS3 cells, which was consistent with the cell growth inhibition assay trend. As the continual expression of the p53 protein in the HeLaS3 cells was especially weak on day 6, the level of growth suppression would appear to be more prominent than in the SiHa cells. There was no p53 expression in the mock-infected cells or those infected with AdCMVLaCZ (Data not shown).

3) Exogenous p53 and E6 expression at transcriptional level

HPV has two transforming genes that encode the E6 and E7 oncoproteins. The E6 can form complexes with p53, and promote its degradation. The reciprocal interaction between the E6 and p53, in the HPV-positive cancer cell lines transfected with AdCMVp53, was evaluated by RT-PCR. As shown in Fig. 4, from the results the HPV-16 E6 and HPV-18 E6 showed a decrease in their expression patterns with increasing time. Furthermore, they both showed the characteristic splice product of the E6 transforming gene, E6', which was amplified by this primer pair. As noted, a differential expression pattern of the E6 existed in each cell. In the CaSkI and HeLa cells, the highly expressed E6 transcript gradually decreased, and was completely inhibited after day 6, whereas in other cells, the E6 expression was detected at low levels, and completely inhibited after day 1. The p53 was evenly expressed in each of the cell lines, and became prominently expressed on days 6 and 7, indicating that the expression of the exogenous p53 may gradually induce E6 exhaustion. The β-actin transcripts were evenly expressed in each of the cell lines. Taken together, the gradual decrease in the E6 expression suggests that the growth suppression in cervical cancer cells is mediated by exhaustion of the E6 due to complexation with the p53 protein.

4) Anti-tumor effects of AdCMVp53 in nude mice

Further study was focused on the CaSkI and SiHa cells, as these two representative types of cell displayed differential growth inhibition patterns toward the AdCMVp53. To investigate whether the overexpression of the p53 protein induces cell-dependent apoptosis, in vivo, the AdCMVp53 was infected into cancer cells-xenografted nude mice, and each tumor measured on days 3, 5, 8, 11, 14, 17, 20 and 23. As shown in

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**Fig. 3.** p53 protein expression in cervical cancer cells after AdCMVp53 infection. Each cell line, infected with AdCMVp53 at 50 MOI, was incubated for 6 days. Cells were harvested on specific days, and cell crude extracts obtained. Ten µg of the proteins were subjected to 10% SDS polyacrylamide gel electrophoresis, and analyzed with the ECL detection method. A: CaSkI; B: SiHa; C: HeLa; D: HeLaS3; E: C33A; F: HT3. Each number indicates the incubation periods: lane 1 (0 days); lane 2 (2 days); lane 3 (4 days); Lane 4 (6 days).

**Fig. 4.** Regulation of the E6 and p53 transcript expressions by the adenovirus p53 in vitro. On a specific day during a 7 day period, 100 ng of each cDNA of the various cervical cancer cell lines were amplified with E6 primers, and separated by agarose gel electrophoresis for analysis: A: CaSkI; B: SiHa; C: HeLa; D: HeLaS3.
Fig. 5. Tumor growth-inhibitory effects of adenovirus p53 in CaSki and SiHa cell-xenografted nude mice. Mice were subcutaneously injected with $10^7$ cells/mouse. Two weeks later, 100 μl of AdCMVp53 (10^9 pfu/ml), AdCMVLacZ (10^9 pfu/ml) and PBS alone were injected into tumors of 7–8 mm in diameter. Seven mice were used for each treatment. Statistical significance was accepted at a p value <0.05, using the paired Student’s test, compared to the PBS control, as indicated by the * character (*, P < 0.05).

Fig. 6. Immunohistochemistry analyses of the AdCMVLacZ and AdCMVp53. The LacZ and p53 expressed in the SiHa cell-xenografted tumor tissue was observed with a microscope on day 2 after the injection of each viral stock, 100 μl (10^9 pfu/ml), into the tumor tissue of the nude mice. For the AdCMVLacZ, blue colored nucleus staining was observed: A (×200); B (×400). For the AdCMVp53, brown colored nucleus staining was observed: C (×200); D (×400).
Fig. 5, in the CaSkii cell-xenografted tumor tissues, the size was decreased to 23.2, 34.3, 33.9, 35.8, 19.1, 20.8, 15.7 and 12.7%, respectively, compared with the AdCMV-LacZ and PBS injection. Also, in the case of SiHa cell-xenografted nude mice, the size reductions on for the same number of days were 27.4, 26.6, 25.2, 28.0, 28.8, 28.7, 28.9 and 27.1%, respectively, although with no statistical significance in the early days. However, it was notable that the levels of tumor growth inhibition in the early stages (for about 11 days) were consistent with the in vitro assay results, where the cell growth inhibition was remarkable in the CaSkii cells. No significant differences in the anti-tumor effect of the PBS and AdCMV-LacZ were observed in this assay.

5) Immunohistochemistry analysis

In the SiHa cells-xenografted nude mice, the immunohistochemistry results showed that the expressions of the p53 protein and LacZ were about 60.5 and 67.7%, respectively, on day 2 (Fig. 6). These results show that the adenovirus-mediated gene transfection was very effective in vivo.

6) p53 and E6 expression in vivo

To evaluate the reciprocal interaction between the p53 and E6 in the SiHa AdCMVp53 transfected cells-xenografted nude mice, RT-PCR was performed and the inhibitory property against the expression of p53 observed in vivo. The results showed that the p53 transcript was highly expressed for several days, but rapidly decreased on day 21 (Fig. 7). In contrast, the expression of E6 transcript started to increase from day 14 and was highly expressed by day 21, which was accompanied by a decrease in the p53 expression. This was consistent with the in vitro E6 and p53 transcript expression patterns. The disappearance of E6 transcript expression only was due to the complex formation with excessive exogenous p53 protein. The -actin was evenly expressed, irrespective of the p53 and E6 expression pattern.

As shown in Fig. 8, in the western blot assay for the SiHa AdCMVp53 transfected cells-xenografted nude mice, the p53 protein was highly expressed for 7 days, but was merely detected on day 14, which was consistent with the p53 transcript expression noted above. As expected, in the cases of either PBS injection or AdCMV-LacZ transfection, no p53 protein was detected.

**DISCUSSION**

The direction of cancer gene therapy has recently focused on the gene-level, where the strategy is to gain an understanding of the biological characteristics of cancer tissues, and then to suppress the growth of cancer cells via improvements in the molecular level therapeutic methods administered to the human body in vivo. However, these methods remain far from ideal in clinical cervical cancer therapies as the effects of gene therapy may occur by a tumor-specific pathway in humans. Moreover, the Food and Drug Administration have recently suspended 27 gene therapy trials involving several hundred patients after learning that two children treated in France had developed a condition resembling leukemia. The temporary halt, the largest such action involving gene therapy trials, was yet another setback to this fledgling field, still shaken by the death, in 1999, of a teenager undergoing gene therapy at the University of Pennsylvania and the first case of leukemia in a French infant last year. It is becoming increasingly clear, even though gene transfer has become more efficient, that another problem could be specific to the treated disease or to the gene used.

In this study, the effect of p53 adenoviral vector on cervical cancer cells was evaluated to gain an insight into the molecular basis of tumor-specific growth inhibition, both in vitro and in vivo. First, in order to validate our experimental approach, the cell growth rates of the CaSkii and SiHa were compared with the previously reported results. Our results were consistent with the fact that in the early days, the SiHa cells grow rapidly, but a few days later, the CaSkii cells grow more than the SiHa cells. On day 6, a stable growth rate was shown with the SiHa compared to the CaSkii cells. Reliable experimental data have shown consistency with the previously reported results, and the validation of the profiling method for studying the tumor-specific pathway by adenoviral p53 delivery.
HPV-16 or -18 positive cervical cancer cells are reported to express only low concentrations of the p53 transcript, due to E6-activated-ubiquitin dependent protease digestion. Our in vitro results showed that after infection with adenovirus p53, the expression of the p53 protein was highly maintained for a few days in the CaSki and HeLa compared to the SiHa and HeLaS3 cells. From the RT-PCR, the p53 transcripts were highly expressed for long periods of time. The expression of the E6 transcript gradually decreased, depending upon the cell type, indicating that although the exogenous p53 was sufficiently expressed in cells, the difference in the expressions between the p53 transcript and protein results from the level of the E6-regulated-ubiquitin-mediated protein degradation. The characteristic splice product of the E6 transforming gene, E6Δ1, was highly detected in all cells (13), but the reciprocal interaction between the E6 and p53 was not quantitative. It was confirmed that the cell-dependent significant growth suppression resulted from the overexpression of the exogenous p53. In particular, the HPV 18-infected cell types were more susceptible to p53-mediated cell growth inhibition than the HPV 16-infected cell types. It was speculated that cells with a smaller HPV copy number might be more susceptible to growth inhibition by adeno-associated viruses (14). In this study, the SiHa cells (1~2 copies of HPV per cell) showed more susceptibility for the first 5 days compared to the CaSki (600 copies) and HeLa (50 copies), with the exception of the HeLaS3 cells (10 copies). However, this was not the case on day 6, where the susceptibility was not dependent on the HPV copy number at all. Conversely, no suppression of the cancer cell growth was observed after infection of the cells with AdCMVlaczZ, supporting the view that the effects were not mediated by the virus itself.

In HPV 16- and HPV 18-infected cells, overexpression of the exogenous p53 may induce E6 exhaustion, which then promotes the cell growth inhibition. The expression of the exogenous p53 is directly related to the inhibition of the NF-κB activity, which is associated with the prevention of apoptosis (15). More recent studies have demonstrated that the combination of p53 therapy, with radiation or chemotherapy, is more effective in suppressing cancer growth compared to a single therapy (16). In p53-mediated apoptosis, apoptotic protease activating factor-1 (APAF-1) plays an important role as a p53 downstream effector, as confirmed by a cDNA microarray (17). In this study, we confirmed the notion that the wild-type p53 is responsible for apoptosis and cell death in cancer cells. Its growth suppression mechanism is expected to occur through the suppression of the G1 phase of the cell cycle by the wild-type p53 gene (18). In particular, when delivered in a virus vector form, the p53 induces G1 arrest in osteoblastoma cells. It has previously reported, by our group, that p53 delivery induced G1 arrest in HeLa cells (19). In contrast, the G2/M phase arrest was caused by the overexpression of p53 in CaSki cells. In particular, S phase arrest also occurred in CaSki cells, resulting in the idea that the arrest phase is dependent on the cervical cancer cell line. In order to obtain a molecular-level view of the cell-specific tumor suppression effect caused by the exogenous p53, the cell cycle checkpoints should be examined further.

It is well known that the expression of the p53 protein is barely detected in tumor tissues without recombinant adenoviral p53 delivery (19). Therefore, as the p53 protein, originating from cancer cell xenografted nude mice, does not exist, or exists at levels below the detection limit, the tumor tissues in immunity-disordered nude mice would be proliferated without any hindrance (20). In this study, the inhibitory properties against p53 showed that the increased expression of the E6 transcript was accompanied by the decrease in the p53 expression in vivo. A possible explanation for this might be that the continual release the E6 oncogene overcomes the suppression of the overexpressed exogenous p53 protein. It has been reported that a single injection of adenovirus p53 suppressed SiHa cell growth for 21 days, which then initiated the cessation of the cell growth inhibition (3). In most adenoviral vector systems, the expression of exogenous gene is transient as the host immune system plays a major role in preventing the sustained expression of foreign genes (9).

It was observed that the expression of the p53 protein, originating from the recombinant adenoviral p53, was increased in cancer lesion sufficiently to overcome the capabilities of the endogenous E6 to bind to the p53 protein (21). The p53 protein expression was detected for a long assay period, consistent with the result previous reported in lung cancer (22). It has been reported that the anti-cancer effect has taken place, via exogenous p53-mediated apoptosis, as the cellular levels of the p53 direct the cell through a pathway leading to the apoptosis at higher p53 expression levels; otherwise, through a pathway leading to growth arrest at lower of p53 expression levels (23). In the CaSki cells, the inhibition of the cell growth was significant in the early days in vitro, whereas in the SiHa cells, the inhibition was not detected for this period. The in vitro results were due to the differential expression patterns of the p53 and E6 transcript, leading actually to different effects on the tumor growth inhibition in vivo. Note that the in vivo results provided for the existence of a cell-dependent growth inhibitory pathway, which was consistent with our previous report (19). It was notable that there was no local recurrence in mice, even though the E6 transcript was highly expressed from day 21. It has been reported that different tumor types might exhibit differential responses to the overexpressed wild-type p53, and that the adenoviral p53 vector is not always cytotoxic to tumor cells (24). It was recently reported that the level of primary receptor existence of the adenoviruses in host cancer cells mainly restricts the therapeutic efficacy of the adenoviral vector, as well as the efficiency of gene transfer, in vivo (25). Thus, an improved strategy for cancer gene therapy depending on either a cancer cell-dependent, or cell-independent, pathway should be studied.

**CONCLUSIONS**

In this study, for the successful medical treatment of cervical cancer, originating from a high-risk HPV infection, understanding of the molecular-level cell-specific growth suppression effects by adenoviral p53 was provided. The p53-mediated apoptosis, of the reciprocally associated E6 oncprotein, can be applied to an advanced strategy for the development of preferential tumor cell-specific delivery and the long-term per-
Persistent gene expression control of p53 in cancer gene therapy.

REFERENCES