Basic Investigations

GROWTH INHIBITION OF HUMAN LARYNGEAL CANCER CELL WITH THE ADENOVIRUS-MEDIATED p53 GENE

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ABSTRACT

Objective: In most laryngeal cancers, the function of p53 gene is down regulated. To explore the potential use of p53 in gene therapy of laryngeal cancer, by introducing wild-type p53 into laryngeal cancer cell line via a recombinant adenoviral vector, Ad5CMV-p53 and analyzing its effects on cell and tumor growth. Methods: A human laryngeal cancer cell line Hep-2 was used. Recombinant cytomegalovirus-promoted adenoviruses containing human wild-type p53 cDNA was transiently introduced into Hep-2 line. The growth suppression of the Hep-2 cells and established s.c. squamous carcinoma model was examined. The p53 protein expression was detected using immunohistochemical analysis. Results: The transduction efficiencies of Hep-2 cell line were 100% at a multiplicity of 100 or greater. The p53 protein expression peaked on day 2 after infection and lasted far 5 days. *In vitro* growth assays revealed cell death following Ad5CMV-p53 infected. *In vivo* studies, Ad5CMV-p53 inhibited the tumorigenicity of Hep-2 cell, and in nude mice with established s.c. squamous carcinoma nodules showed that tumor volumes were significantly reduced in mice that received peritumoral infiltration of Ad5CMV-p53. Conclusion: Adenovirus-mediated antitumor therapy carrying the p53 gene is an efficient method to inhibit laryngeal cancer growth. Transfection of laryngeal cancer cells with the wild-type p53 gene via Ad5CMV-p53 is a potential novel approach to the therapy of laryngeal cancer.

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Most malignancies are disease generated with a process of genetic alteration. Based on this theory, new approaches to cancer therapy are being developed. One of these is genetherapy. Among the genes having therapeutic potential for cancer treatment, the p53 tumor suppressor gene has been most extensively studied.[1] Wild-type p53 has been shown to be involved in transcirptional regulation. It arrests cells at the G1/S checkpoint, blocks DNA replication and induces apoptosis.[2] Recent studies have shown that wild-type p53 gene can suppress the growth of some human cancer cell lines.[3]

In the present study, we introduced the wild-type p53 gene into laryngeal cancer cell via the recombinant adenoviral vector, Ad5CMV-p53, to determine its effects on growth of human laryngeal cancer cell Hep-2.

MATERIALS AND METHODS

Cell Line and Culture Condition

Human laryngeal cancer cell line Hep-2 was obtained from our institute. Cells were growth in DMEM with 5% fetal bovine serum (FBS).

Mice

BALB/c nude mice were obtained from The Beijing Medical University and were re-derived and maintained in The Military Academy of Medical Sciences.

Recombinant Adenovirus Production

The recombinant p53 adenovirus containing a

cytomegalovirus (CMV) promoter, wild-type p53 cDNA, and SV40 Polyadenylation (PA) signal in an expression cassette inserted into the E1-deleted region. The defective recombinant control adenovirus (Ad5CMV-polyA) was constructed the some way but does not contain any functional gene between the promotor and the SV40 polydenylation sequence.[3,4] Virue stocks were kindly provided by Dr. Zhang Weiwei (M.D., Anderson Hospital, Houston, USA). Viral stocks were propagated in 293 cells. Cell were harvested 36–40 h after infection, pelleted, resupended in phosphate-buffered saline (PBS), and lysed by the three-time freeze-thaw method. Cell debris was removed and the virus was purified by CsCl gradient centrifugation. The concentrated virus was dialyzed, aliguated, and stored at -80°C.

Cell Growth Assay

Cells were plated at a density of 1×10^4/well in 24 well plates in triplicate. They were infected with either Ad5CMV-p53 or the Ad5CMV-polyA cells were harvested and counted at each point of the dose and the dose and the date. Cell viability was determined by trypan blue exclusion.

Immunohistochemical Analysis

The infected cell monolyers were fixed with 100% alcohol. Immunohistochemical staining was performed. The primary antibody was the anti-p53 antibody DO7 (DAKO), and the secondary antibody was an avidin-antibody complex. Biotinylated horseradish peroxidase ABC complex reagent was used to detect the antigen-antibody complex.

Tumorigenicity Assays

Hep-2 cells were infected with Ad5CMV-p53 and Ad5CMV-polyA at 50 MOL (PFU/cell). An equal amount of cells were treated with medium as a mock infection. 18 h after infection, the treatment cells were harvested. For each treatment, 5×10^6 cells in a volume of 0.1 ml PBS were injected to three separate flaps of BALB/C nude mice. Tumor formation was evaluated at the end of a 6-week period.

Inhibition of Tumor Growth *in Vivo*

The effect of Ad5CMV-p53 on established s.c. tumor nodules was determined in nude mice. Briefly, three separate flaps were elevated on each animal, and 5×10^6 cells in 0.1 ml of PBS were injected into each flap using a blunt needle. After 6 days, the flaps of the animals were injected for the delivery of 0.1 ml of: (a) Ad5CMV-p53 (1×10^8 PFU) in the right posterior flap; (b) Ad5CMV-polyA (1×10^8 PFU) in the left posterior flap; and (c) PBS in the anterior flaps. All injection sites had developed s.c. visual and palpable nodules before treatment was administered. Treatments were given once per three days for 6 times. Tumor volumes were calculated by assuming a spherical shape, with the average tumor diameter calculated as the square root of the product of cross-sectional diameters.

RESULTS

Immunohistochemical Analysis

To obtain a high expression of p53 protein, human CMV promoter was used o drive the wild-type p53 cDNA.. Immunohistochemical staining for p53 protein expression in Hep-2 cell confirms that most of the cells stained positive for p53 at 100 MOI (data not shown). The mock and Hep-2 cells infected by Ad5CMV-polyA did not show any detectable expression of p53 protein. This indicated that the exogenous p53 cDNA was successfully transducted into cells and efficiently transcribed. The high transduction efficiency of this vector was also found in other cell lines examined previously.

Effects of Exogenous p53 on Hep-2 Cell Growth *in Vitro*

The growth of the Ad5CMV-p53-infected cells was greatly suppressed at 100 MOI when estimated by cell count assay (Figures 1, 2). Cell growth assays were reproducible in three repeated experiments.

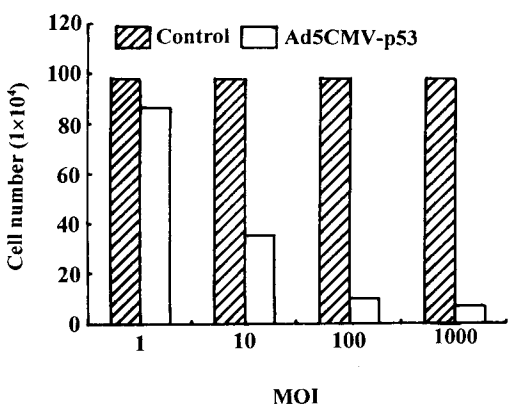

Fig. 1. Inhibition of the growth of Hep-2 cells by Ad5CMV-p53 at different MOI by cell count assay. The means of cell counts for triplicate wells 4 days after infection were plotted.

Ad5CMV-p53 Overexpression Inhibited Hep-2 Tumor Development *in Vivo*

To examine whether the Ad5CMV-p53 virus can inhibit tumorigenicity of human laryngeal cancer cells, nude mice were injected subcutaneously with Hep-2 that

were exposed to Ad5CMV-p53 or Ad5CMV-polyA (100 MOI) 18 h prior to tumor cell incubation Ad5CMV-p53-treated cells did not develop tumors during a 3-week observation period (Table 1).

Table 1. Effect of Ad5CMV-p53 on tumorigenicity in nude mice

Treatment	No. of tumors/No. of mice (%)
Control	7/7 (100%)
Ad5CMV-polyA	6/7 (86%)
Ad5MCV-p53	0/7 (0)

Effects of Exogenous Wild-type p53 on Hep-2 Cell Growth *in Vivo*

To address the feasibility of p53 gene therapy for established tumors, the efficacy of Ad5CMV-p53 in inhibiting tumor growth was evaluated in Hep-2 tumor-bearing nude mouse model. The mice received six intratumoral injections of PBS only, Ad5CMV-polyA, or Ad5CMV-p53. In the mice treated with PBS or Ad5CMV-polyA, Hep-2 cell tumors continued to grow rapidly throughout the treatment, whereas those treated with Ad5CMV-p53 grew at a greatly reduced rate (Figure 3). At the end of experiment, Ad5CMV-p53 had reduced tumor size by 99.4% compared with PBS only, whereas Ad5CMV-polyA did not significantly reduce tumor size (Table 2).

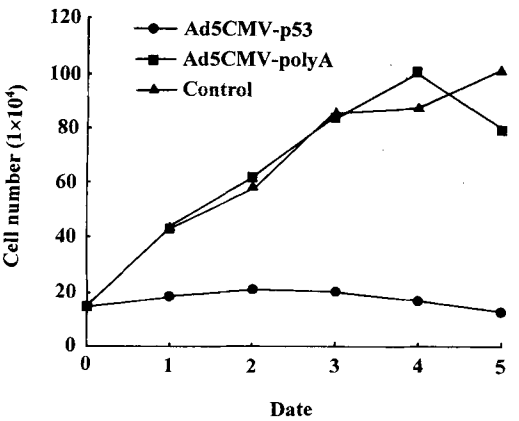

Fig. 2. Time course of the growth inhibition of Hep-2 cells by Ad5CMV-p53 and Ad5CMV-polyA by cell count assay.

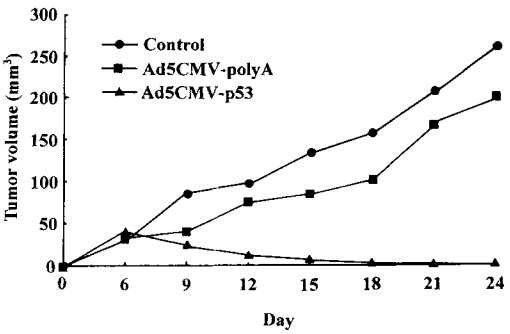

Fig. 3. Effect of treatment with Ad5CMV-p53 on tumor growth of Hep-2 cells in nude mice.

Table 2. Effect of Ad5CMV-p53 on established in vivo tumor growth in nude mice

Treatment	No. of tumors/No. of mice	Mean volume (mm^3, $\bar{x}\pm s$)[a]	%
PBS	7/8	259.40±61.95	100
Ad5CMV-polyA	5/8	198.33±27.9[b]	76.45
Ad5CMV-p53	7/8	1.54±1.42[c,d]	0.59

DISCUSSION

The adenovirus-based vector has emerged as a leading candidate for *in vivo* gene therapy in recent years. Adenovirus vectors possess several properties advantageous for gene therapy over other vector system. Recombinant adenoviruses can be grown to high titers, can infect most cell types and cells in all phases of the cell cycle, have a high single infection efficiency, and are free of any obvious cytopathic effect.[3] Tumor suppressor gene p53 is among the genes with the greatest therapeutic potential for cancer treatment. In this study, recombinant adenovirus that expresses the wild-type p53 was generated. Because wild-type p53 protein is considered a potent growth inhibitor for malignant human tumor cell lines, we first tested the efficacy of wild-type p53 adenovirus in the human laryngeal cancer line Hep-2 both $^{\text{in vitro}}$ and $^{\text{in vivo}}$.

Immunohistochemical analyses and cell count assay demonstrated that production of the p53 protein were significantly increased in Ad5CMV-p53-infected cells when compare with control cells, suggesting that the exogenous p53 mRNA may be efficiently translated. At more that 100 MOI, the growth of Hep-2 cells was greatly suppressed and all infected cells were stained positive for p53. These results were similar to these of the previous study of cancer cell lines.[5,6] The *in vitro* growth of laryngeal cancer cells after transduced with the wild-type p53 gene was significantly inhibited when compared with mock-infected and Ad5CMV-polyA-infected cells, suggesting that these results were not mediated by the virus itself.

Adenovirus-mediated p53 expression has been shown to suppress the growth of established head and neck tumor cells in nude mice and to prevent the growth of these tumor cells in a model to inhibit residual tumor

burden.[7,8] Our studies with adenovirus p53 therapy of established laryngeal cancer tumors also support these findings and show that p53 adenoviral vectors may be useful to inhibit the growth of a variety of tumors.

Laryngeal cancer is particularly well suited for gene transduction strategies. First, its location in the upper respiratory tract allows easy access for both vector delivery and assessment of response. Second, a degree of tissue specificity of gene transduction can be achieved by direct injection of the vector into the tumor mass. Finally, metastases mostly occur late in laryngeal cancer progression, making local and regional disease responsible for most of the morbidity and mortality. Therefore, any improvement in local and regional control potentially result in benefits for patients.

In conclusion, these findings indicate that the overexpression of p53 protein using these vectors may promote efficient tumor cell death. Our studies lend support to the application of p53 as a gene therapy agent in head and neck cancer.[9] The use of these vectors may be a potent tool for reducing tumor growth and may be a potential therapy for treatment of laryngeal cancer.

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