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Advances in Brief

Adenovirus-mediated Wild-Type *p53* Expression Induces Apoptosis and Suppresses Tumorigenesis of Prostatic Tumor Cells

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Abstract

The use of replication-deficient adenoviral vectors in gene therapy may become a powerful method to achieve efficient but safe transfer of antitumor agents. Introduction of the wild-type *p53* gene into tumor cells has, in general, been associated with growth suppression. In this study, infection of androgen-independent human prostate Tsu-pr1 cells lacking functional *p53* alleles resulted in high levels of p53 protein within 10–15 h. Cells infected with AdCMV.p53 detached from the substratum, condensed, and exhibited fragmentation of nuclear DNA into nucleosomal units consistent with the process of apoptosis. These effects were evident within 24 h after infection, and the majority of cells had undergone apoptosis by 48 h, whereas cells infected with AdCMV.NLSβGal continued to proliferate. Uninfected or AdCMV.NLSβGal-infected Tsu-pr1 cells formed tumors in nude mice within 3 weeks after implantation, whereas AdCMV.p53-infected cells failed to form tumors during this period. Therefore, adenoviral-mediated antitumor therapy using the *p53* gene is an efficient method to inhibit prostate tumor growth, and agents that target the cellular programmed cell death pathway may be useful in clinical applications.

Introduction

Prostate cancer is the most diagnosed cancer in men and the second leading cause of cancer deaths in the United States (1). Great progress has been made toward the treatment of prostate cancer since the recognition that androgen is required for prostate development (2) and tumor growth (3). Castration has been routinely performed to treat prostate cancer for many years (3). Chemical castration using estrogens (4), luteinizing hormone-releasing hormone analogues (5), and anti-androgens (6) has been demonstrated to have some effects on the growth of prostate cancer. However, when prostate tumors progress to stage D metastatic disease, none of these treatments is effective (7). Recent data have demonstrated that metastases of prostate cancer and prostate cancer cell lines frequently contain mutations and deletion of *p53*, a tumor suppressor gene (8–10), although primary tumors have few *p53* mutations (11, 12). *p53* has been implicated as a molecule of central importance in the maintenance of genomic stability to prevent mutation or deletion of functional genes because of its involvement in DNA damage-induced G_1 cell cycle arrest, apoptosis, and gene amplification (13). Direct induction of melanoma cell apoptosis by *p53* (14) and mediation of other apoptotic stimuli by *p53* (15) indicated the potential application of wild-type *p53* in prostate cancer therapy.

Replication-deficient adenovirus vectors (AdCMV.p53[2] and AdCMV.NLSβGal coding for human *p53* and bacterial β-galactosidase, respectively) were chosen in these experiments because gene transfer to cells at any stage of the cell cycle is possible and because transient gene expression without integration into the genome assures the safety of the therapy (16). In the present study, the expression pattern of adenovirus-mediated *p53* in human prostate Tsu-pr1 cells was investigated. These cells are *p53* deficient due to loss of one allele and mutation of the second allele (17). Our studies extend the findings that *p53* expression inhibits cell growth and show that overexpression of wild-type *p53* with an adenoviral vector results in the programmed cell death of prostate tumor cells and the inhibition of tumor cell growth *in vivo*.

Materials and Methods

Cell Culture and Adenoviral Construction. The prostate carcinoma (Tsu-Pr1) cell line, obtained from Dr. John Isaacs (Johns Hopkins Oncology Center), was maintained in RPMI 1640 supplemented with 10% FBS, penicillin streptomycin antibiotic, L-Glutamate, and nonessential amino acids (NIH Media Unit, Bethesda, MD). Confluent cells were used for the experiment. Adenovirus containing wild-type *p53*, AdCMV.p53 (obtained from Dr. Bert Vogelstein, Johns Hopkins Oncology Center), is a replication-deficient adenoviral vector containing a CMV promoter and a recombinant human wild-type *p53*. Recombinant human wild-type *p53* cDNA was inserted into a replication-deficient adenovirus vector containing the CMV promoter for efficient expression (16). The virus was amplified in 293 packaging cells containing the genes for viral replication. Adenovirus containing the *LacZ* gene, AdCMV.NLSβGal, coding for the bacterial enzyme β-galactosidase, were prepared as a control.

DNA Fragmentation and Cell Viability. Cells in serum-free RPMI were infected with either AdCMV.p53 or AdCMV.NLSβGal for 1 h by using 30 plaque-forming units/cell. Cells were washed and maintained in serum-free RPMI 1640 for 2 days. Floating cells were collected by centrifugation and combined with attached cells harvested in 100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.5% SDS, and 0.1 mg/ml proteinase K, pH 8.0 (digestion solution). After overnight incubation at 50°C, the cell lysates were extracted with phenol/chloroform and the DNA in the aqueous phase was precipitated and resuspended in Tris-EDTA buffer. After digestion with 5 μg/ml RNase A for 1 h, DNA was precipitated, resolved by electrophoresis on a 1.5% agarose gel, and visualized by ethidium bromide staining.

The conversion of the tetrazolium salt MTT (United States Biochemical) to formazan by the cells was used, with some modification, to assess cell viability (18). In brief, confluent cells were infected with AdCMV.p53 or AdCMV.NLSβGal adenoviral vectors in RPMI 1640 with 2% FBS for 1 h. Cells were washed with serum-free RPMI 1640 and maintained in serum-free medium. At different times, cells were incubated with MTT (0.5 mg/ml) at 37°C for 3 h. Cells were scraped and collected, and dimethylsulfoxide was added to dissolve the insoluble blue product. The absorbance at 550 nm was measured on a spectrophotometer.

SDS-PAGE and Immunoblotting. After treatment, attached cells were washed with ice-cold PBS and harvested in wash buffer [10 mM Tris, 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml Aprotinin (Sigma Chemical Co., St. Louis, MO)] by scraping. Floating cells collected by centrifugation were combined with attached cells and pelleted by centrifugation. The pellets were resuspended in a minimal amount of lysis buffer (10 mM Tris, 1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml Aprotinin), and

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[2] The abbreviations used are: AdCMV.p53, cytomegalovirus promoter p53 adenoviral vector; AdCMV.NLSβGal, cytomegalovirus promoter β-galactosidase adenoviral vector; MTT, 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide; CMV, cytomegalovirus.

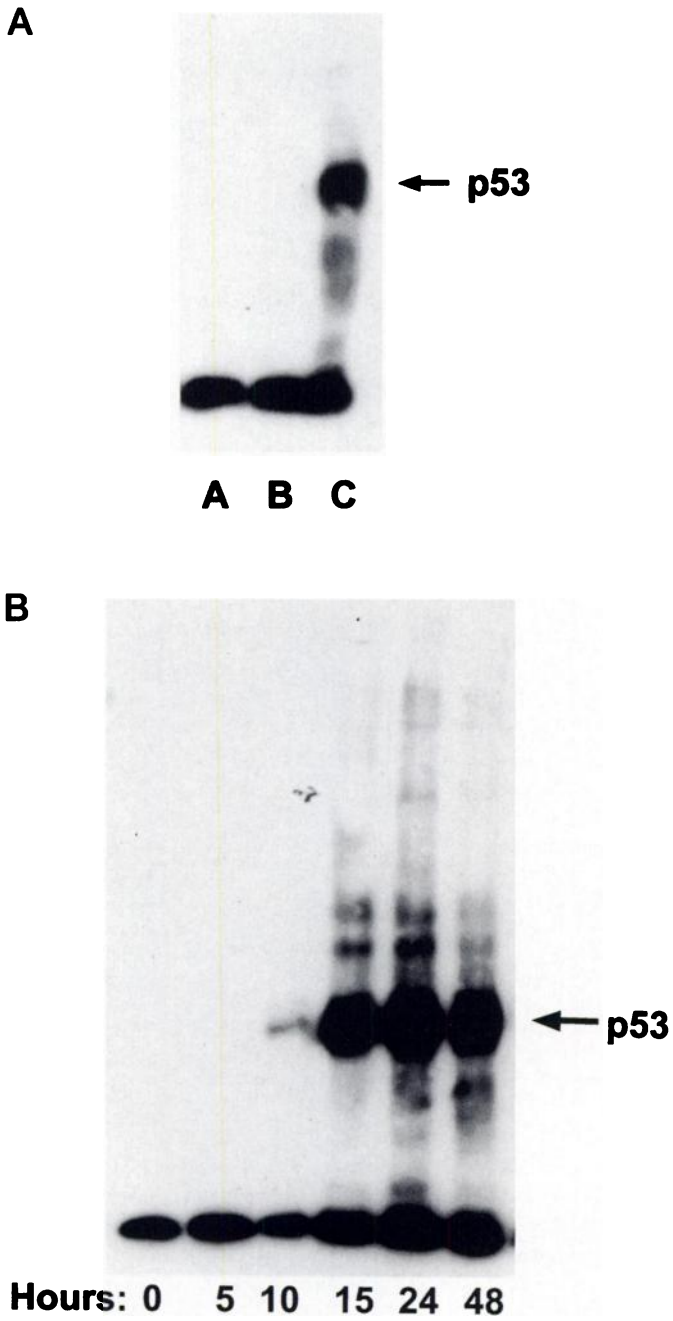

Fig. 1. Adenovirus-mediated p53 expression in Tsu-pr1 prostate tumor cells. *A*, expression of p53 protein in uninfected (*Lane A*), AdCMV.βGal-infected (*Lane B*), or wild-type p53 AdCMV.p53-infected (*Lane C*) cells after 24 h. Western blotting was performed as described in "Materials and Methods." *B*, time course of p53 protein expression in adenovirus-infected cells.

the DNA was disrupted by passing through a 27-gauge needle. The concentration of protein was determinated by BCA assay (Bio-Rad). Proteins were separated on a 10% SDS slab gel and visualized by staining with Coomassie. After separation by SDS-PAGE, proteins were transferred to Immobilon-P transfer membranes (Millipore Corp.). The membranes were blocked with 1% BSA and probed with mAb against wild-type human *p53* (Santa Cruz) overnight at 4°C. After extensive washing, enhanced chemiluminescence mixture reagents (Amersham) were added to the membrane for 1 min. The membrane was covered with Saran wrap and exposed to X-ray film.

Tumorigenesis in Vivo. Tsu-Pr1 cells were suspended in Matrigel at a concentration of 1×10^7 cells/ml. Matrigel was prepared from the Engelbreth-Holm-Swarm tumor following procedures described previously (19). Cell suspension (0.5 ml) was injected into each nude mouse. Tumor formation was

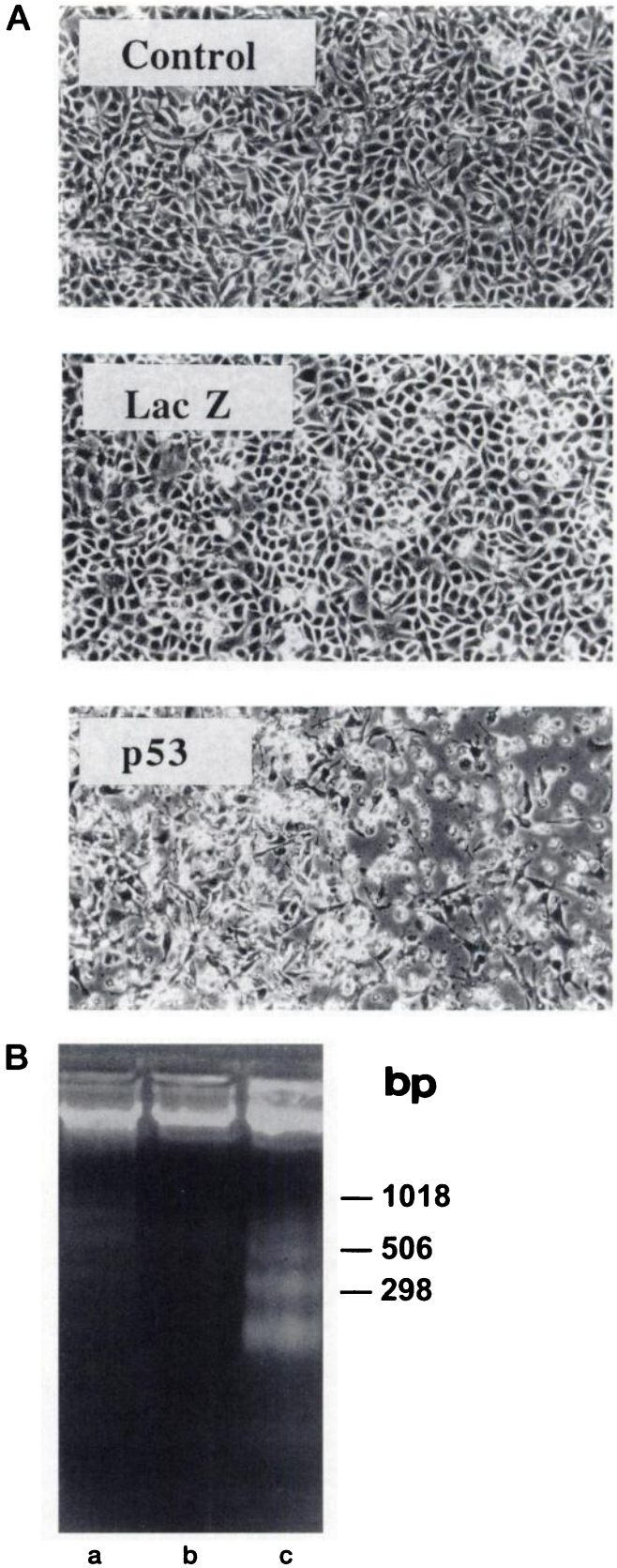

Fig. 2. Induction of apoptosis by adenovirus-mediated p53 expression. *A*, morphology of uninfected Tsu-pr1 cells (control) or cells infected with adenovirus expressing the βGal (*LacZ*) or wild-type *p53* gene. *B*, agarose gel electrophoresis of DNA isolated from Tsu-pr1 cells uninfected (*a*), or infected with adenoviral vectors expressing βGal (*b*), or wild-type *p53* (*c*). All analyses were performed 48 h after infection.

observed 3 weeks after the injection, and tumor sizes were estimated with calipers.

Results and Discussion

Expression of Adenovirus-mediated Wild-Type *p53* in Tsu-pr1 Cells. Tsu-pr1 cells contain only one allele of *p53* on chromosome 17p, with a mutation at codon 126 from TAC to TAG resulting in a stop codon. Accordingly, no p53 protein was detectable by immunoblotting (Fig. 1*A*, *Lane A*) consistent with previous reports (17). It has been shown that wild-type *p53* can suppress the growth of Tsu-pr1 cells (17). Tsu-pr1 cells were infected with AdCMV.p53 and expressed very high levels of p53 after 24 h (Fig. 1*A*, *Lane C*), but no p53 protein was detected in the AdCMV.NLSβGal-infected cells. Detectable levels of p53 protein were observed 10 h after infection and reached a maximum after 15 h (Fig. 1*B*). High levels of p53 protein were maintained in these cells for up to 2 days after infection. These data are consistent with CMV-driven expression of other genes engineered into adenoviral vectors and confirm other findings that in rapidly dividing cells, gene expression declines several days after infection.

p53 Expression Induces Apoptosis in Tsu-pr1 Cells. It has been shown that p53 induces growth arrest, and, in some cases, eventually causes either necrosis or apoptosis (13). Forty-eight h after infection, high expression of p53 in Tsu-pr1 cells was associated with cell death (Fig. 2*A*). Cell condensation and detachment occurred in p53-infected cells, whereas βGal-infected cells retained a morphology similar to uninfected cells. Nuclear DNA from cells infected with AdCMV.p53 was isolated and showed a distinctive laddering pattern in agarose gel electrophoresis. DNA from uninfected or AdCMV.NLSβGal-infected cells showed only a band at a high molecular weight corresponding to intact DNA (Fig. 2*B*). These results indicate that the cell death induced by wild-type *p53* is apoptosis with nucleosomal fragmentation of DNA. The dynamics of cell death were detected with the MTT assay, which allows the quantitation of viable cells. In the absence of serum, uninfected and βGal-adenovirus-infected Tsu-pr1 cells continued to proliferate. Proliferation was suppressed by wild-type *p53*. The suppression of growth by p53 was followed by a sharp decrease in the number of viable cells. After 72 h, nearly all of the p53 adenovirus-infected cells had died (Fig. 3).

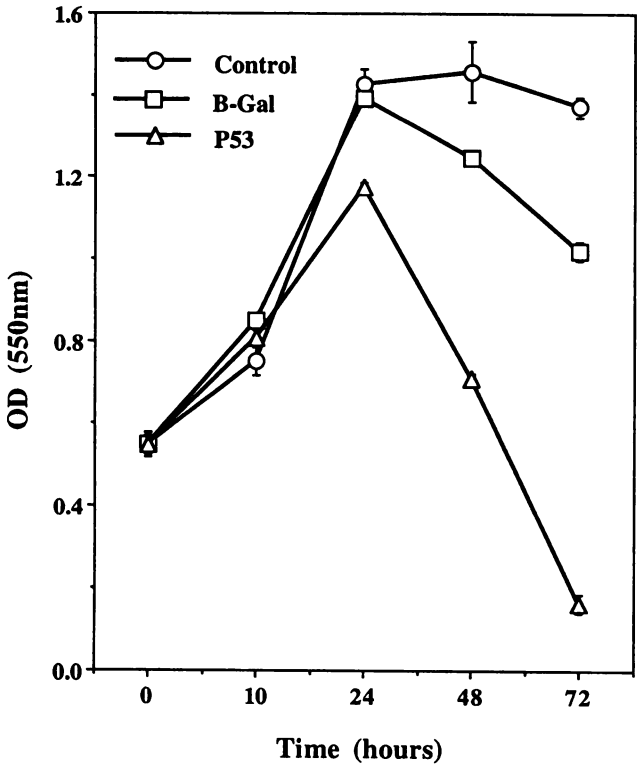

Fig. 3. Effect of adenoviral p53 expression on cell viability. Tsu-pr1 cells were infected with adenoviral vectors expressing the βGal (□) or the wild-type *p53* genes (△) or were uninfected (○). Cells were infected for 1 h and then incubated in serum-free medium. Control cells remained in serum-free medium during the course of the experiment. *OD*, absorbance.

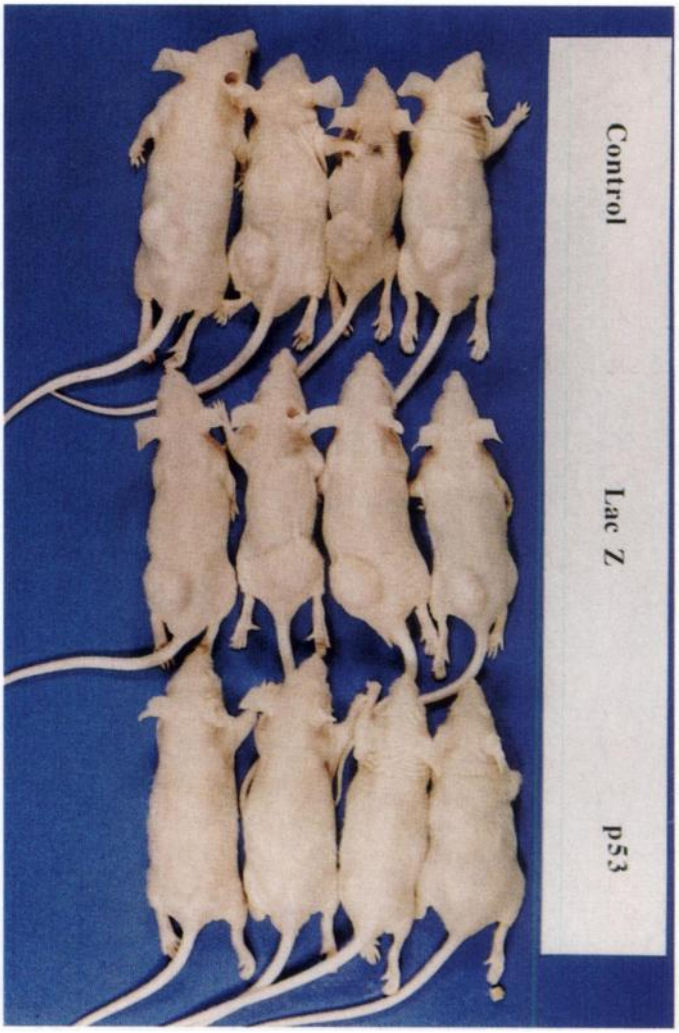

Fig. 4. Adenovirus p53 expression and tumor growth *in vivo*. Tsu-pr1 cells were infected with AdCMV.βGal (*LacZ*) or AdCMV.p53 coding for wild-type *p53* for 1 h and then transplanted into nude mice. Uninfected cells were used as control.

Although transfection of tumor cells with the wild-type *p53* gene has been shown to directly induce apoptosis, the role of endogenous p53 expression in apoptosis in the prostate gland after androgen ablation is controversial (20, 21). Previous transfection studies of prostate tumor cells with p53 cDNA showed that p53 inhibited thymidine incorporation, consistent with the results shown here and consistent with the growth arrest properties of wild-type *p53* (12).

Wild-Type Adenoviral *p53* Expression Suppresses Tumorigenesis. To assess the potential of AdCMV.p53 for treatment of prostate cancer, the effect of wild-type *p53* expression on the growth of Tsu-pr1 cells *in vivo* was investigated. Cells were infected with AdCMV.p53 or AdCMV.NLSβGal and then injected s.c. into nude mice. After 3 weeks, uninfected cells formed tumors in nine of ten mice. The tumor size was in the range of 9–18 mm in diameter. One of the mice exhibited metastases in the lymph node of the neck. Similar tumor formation was observed in the mice injected with cells

infected with AdCMV.NLSβGal. However, only 1 of 10 of mice injected with cells infected with AdCMV.p53 formed a tumor with a size <5 mm in diameter (Fig. 4). These results clearly demonstrate that adenovirus-mediated p53 expression can very efficiently suppress tumor growth.

Adenovirus-mediated p53 expression has been shown to suppress the growth of established head and neck tumor cells in nude mice (22) and to prevent the growth of these tumor cells in a model to inhibit residual tumor burden (23). Our studies with adenovirus p53 therapy of established melanoma tumors (14) also support these findings and show that p53 adenoviral vectors may be useful to inhibit the growth of a variety of tumors. Transfection studies of human prostate tumor cells also resulted in the inhibition of tumor growth in soft agar (17). Our studies suggest that adenoviral-mediated wild-type *p53* overexpression may result in the elimination of tumor cells by apoptosis, thus reducing the tumor burden.

In conclusion, these findings indicate that the overexpression of p53 protein using these vectors may promote efficient tumor cell death. The use of these vectors may, therefore, be a potent tool for reducing tumor growth *in vivo* and may be a potential therapy for treatment of androgen-independent prostate cancer (24).

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