Induction of Chemosensitivity in Human Lung Cancer Cells in Vivo by Adenovirus-mediated Transfer of the Wild-Type p53 Gene

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Abstract

Recombinant adenovirus-mediated transfer of the wild-type p53 gene into monolayer cultures or multicellular tumor spheroids of human non-small cell lung cancer cell line H358, which has a homozygous deletion of p53, markedly increased the cellular sensitivity of these cells to the chemotherapeutic drug cisplatin. Treated cells underwent apoptosis with specific DNA fragmentation. Direct injection of the p53 adenovirus construct into H358 tumors s.c. implanted into nu/nu mice, followed by i.p. administration of cisplatin, induced massive apoptotic destruction of the tumors. These results support the clinical application of a regimen combining gene replacement using replication-deficient wild-type p53 adenovirus and DNA-damaging drugs for treatment of human cancer.

Introduction

The biochemical features of programmed cell death (apoptosis) show a characteristic pattern of DNA fragmentation resulting from cleavage of nuclear DNA. Recent studies have demonstrated that induction of apoptosis by chemotherapy drugs or ionizing radiation may be related to the status of the p53 gene and that DNA-damaging stimuli are able to elevate intracellular p53 protein levels in cells that are in the process of apoptosis (1–5). Inhibition of the cell cycle at G1 by increased levels of the wt-p53 protein allows more time for DNA repair; if optimal repair is impossible, p53 may trigger programmed cell death. Thus, p53 may contribute to the induction of apoptotic tumor cell death by chemotherapeutic agents.

Inactivation of the p53 gene by missense mutation or deletion is the most common genetic alteration in human cancers (6, 7). The loss of p53 function has been reported to enhance cellular resistance to a variety of chemotherapeutic agents (8). Our preliminary experiments showed that human NSCLC H358 cells, in which both alleles of p53 are deleted, were resistant to chemotherapeutic drugs, whereas cell line WTH226b, which has endogenous wt-p53, readily showed apoptotic cell death after treatment with CDDP and etoposide. Therefore, we sought to determine whether the introduction of the wt-p53 gene into H358 cells by an adenoviral vector could increase the sensitivity of the cell to the DNA-cross-linking agent CDDP in vitro and in vivo.

Materials and Methods

H358 cells were kindly provided by A. Gazdar and J. Minna of the University of Texas Southwestern Medical Center, Dallas, Texas (9).

Adenovirus Vectors. The construction and identification of Ad-p53 or Ad-Luc were reported previously (10). Briefly, the p53 expression cassette that contains human cytomegalovirus promoter, wt-p53 cDNA, and SV40 early polyadenylation signal was inserted between the XbaI and ClaI sites of pXCI.1. The p53 shuttle vector and the recombinant plasmid pJM17 were cotransfected into 293 cells (Ad5-transformed human embryonic kidney cell line) by a liposome-mediated technique. The culture supernatant of 293 cells showing the complete cytopathic effect was collected and used for subsequent infections. The control Ad-Luc virus was generated in a similar manner. Ad-p53 and Ad-Luc viruses were propagated in 293 cells. The presence of replication-competent virus was excluded by HeLa cell assays. The viral titers were determined by plaque assays (11).

Detection of Nucleosomal DNA Fragmentation. DNA was isolated from parental, Ad-Luc-infected, and Ad-p53-infected cells that did or did not receive CDDP treatment, by incubating cells at 55°C for 6 h in lysis buffer [50 mM Tris-HCl (pH 8.0), 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate, and 50 μg/ml proteinase K]. DNA was extracted twice with equal volumes of phenol and once with chloroform-isooamylalcohol (24:1) and then precipitated in ethanol. Samples were subjected to electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

Results and Discussion

H358 cells were transduced in vitro with the human wt-p53 cDNA by exposure to Ad-p53. Western blot analysis showed a high level of wt-p53 protein expression as early as 24 h after infection with Ad-p53, but no wt-p53 was detected in parental (uninfected) cells or control cells infected with Ad-Luc (data not shown). Concurrent immunohistochemical evaluation demonstrated detectable wt-p53 protein in more than 80% of infected cells, suggesting that the transfer and expression of p53 by Ad-p53 was highly efficient (data not shown).

Continuous exposure of Ad-p53-infected H358 cells to CDDP reduced their viability rapidly, whereas significant cell death for parental and Ad-Luc-infected cells occurred only after 72 h of exposure to CDDP (Fig. 1A). Loss of viability was greatly enhanced in
not proliferate rapidly in response to exogenous TGF-α. The finding that exposure to CDDP reduced the size of H358 spheroids infected with Ad-p53 by inducing apoptosis on the surface suggests that Ad-p53 infects nonproliferating cells and that CDDP initiates the apoptotic process in quiescent cells.

In an effort to determine the efficacy of a combination of gene replacement therapy and chemotherapy in human cancer, we examined whether sequential administration of Ad-p53 and CDDP could induce apoptosis in vivo. Following 3 days of direct intratumoral injection of Ad-p53 or i.p. administration of CDDP, H358 tumors implanted s.c. into nu/nu mice showed a modest slowing of growth.

**Fig. 1.** The effects of (A) continuous or (B) 24-h exposure to CDDP on the growth rates of parental, Ad-Luc-infected, and Ad-p53-infected H358 cells. H358 cells (1.5 × 10⁵ cells/well) were seeded in duplicate on a 24-well plate. After 24 h, 100 μl of medium, Ad-Luc viral stock (10⁵ PFU/ml), or Ad-p53 viral stock (10⁵ PFU/ml) were added. Following an additional 24-h incubation, the medium that contained virus was replaced with fresh medium that contained 10 μg/ml of CDDP. Cells were exposed to CDDP (A) continuously or (B) for 24 h followed by recovery in drug-free medium. Cells that remained as an attached monolayer were assessed for viability over 5 days by measuring trypan blue uptake. The day 5 cell number for the Ad-p53:CDDP group differs significantly from all other groups for both A and B (P < 0.05 by Student’s t test). C, effects of different concentrations of CDDP on the viability of Ad-p53-infected H358 cells. After 24-h exposure to the Ad-Luc or Ad-p53 virus, cells were treated with 0, 10, or 100 μg/ml of CDDP for 24 h and then assessed for viability. Points, mean; bars, SE.

**Fig. 2.** A, nucleosomal DNA fragmentation in Ad-p53-infected H358 cells exposed to CDDP. Cells were infected and treated with CDDP for 24 h as described in the legend to Fig. 1. B-G, H358 cells were grown on chamber slides, infected with Ad-p53 for 24 h, treated with CDDP for an additional 24 h, and fixed for in situ labeling of DNA fragmentation. Pictured are parental H358 cells (B) without or (C) with CDDP; Ad-Luc-infected cells (D) without or (E) with CDDP; and Ad-p53-infected cells (F) without or (G) with CDDP. Arrowhead, an example of darkly stained nuclear fragments. Bar, 100 μm.
Fig. 3. Effect of the combination of Ad-p53 infection with CDDP treatment on H358 tumor spheroids. A, Multicellular tumor spheroids of H358 cells were prepared as described previously (9). On day 0, spheroids with a diameter of 150-200 μm were placed in a 24-well agar-coated plate and exposed to Ad-p53 or Ad-Luc for 24 h. On day 1, medium with 10 μg/ml of CDDP was added following removal of virus-containing medium. On day 2, after a 24-h incubation, the overlay was replaced with 1 ml of fresh, drug-free medium. The perpendicular diameters were measured using an inverted microscope. The relative volume change was calculated according to the formula $a^2 \times b_1^3 \times b_2$, where $a$ and $b$ are the smallest and largest diameters of the spheroid, respectively, and $a_1$ and $b_1$ are the diameters on day 1. Only the relative volume of the Ad-p53/CDDP spheroids is significantly less ($P < 0.05$ by Student's $t$ test) than the control group (C). B–E, in situ dUTP labeling with TdT for detection of apoptosis. H358 spheroids were fixed on day 3 and stained as described in "Materials and Methods." B, control untreated spheroid; C, spheroid treated with CDDP; D, Ad-p53-infected spheroid; and E, Ad-p53-infected spheroid treated with CDDP. Bar, 100 μm.

However, if Ad-p53 and CDDP were simultaneously administered, tumors partially regressed, and the tumor size remained statistically significantly smaller than those in any of the other treatment groups. The growth inhibitory effect was even more pronounced after two treatment cycles (Fig. 4A). Histological examination revealed a massive destruction of tumor cells in the area where Ad-p53 was injected in mice treated with CDDP. In situ staining demonstrated many apoptotic cells around acellular spaces (Fig. 4, B–E). In contrast, tumors treated with CDDP alone or Ad-p53 alone showed neither acellularity nor apoptotic areas.

This report describes a novel strategy for human gene therapy combined with conventional chemotherapy using a DNA cross-linking agent. Tumor cell resistance to chemotherapeutic drugs represents a major problem in clinical oncology. NSCLC accounts for at least 80% of the cases of lung cancer; patients with NSCLC are, however, generally unresponsive to chemotherapy (18). One goal of current cancer research is to find ways to improve the efficacy of gene replacement therapy for cancer by investigating interaction between the gene product and chemotherapeutic drugs. The herpes simplex-thymidine kinase (HS-tK) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (19). The HS-tK gene product is an exogenous viral enzyme, whereas the wt-p53 protein is expressed in normal tissues, suggesting that the modulation of chemoresistance by alterations in wt-p53 expression might be an alternative approach using a pathway mediated by an endogenous genetic program.

An adenovirus system has potential advantages for gene delivery in vivo, such as ease of producing high titer virus, high infection efficiency, and infectivity for many types of cells. The stability and duration of expression of the introduced gene are still controversial, however. For chemo-gene therapy, the levels of expression and the high infectivity may be more significant than the duration of expression because drugs can kill infected cells within several days. The increase in p53 levels in cells that are sensitive to
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Fig. 4. Induction of apoptosis by CDDP after in vivo infection with Ad-p53. H358 cells (5 x 10^6) in 0.1 ml Hank's balanced salt solution were injected s.c. into the right flank of BALB/c female nude mice. Thirty days later, 200 μl of medium alone or medium containing Ad-Luc (10^10 PFU/ml) or Ad-p53 (10^10 PFU/ml) were injected into tumors with a diameter of 5–6 mm. Intratumoral injection (100 μl) and peritumoral injection in two opposite sites (50 μl each) were performed. CDDP (3 mg/kg) or control physiological saline was given intraperitoneally. A, tumor volume changes. The tumors were measured with calipers in two perpendicular diameters without the knowledge of the treatment groups, and a tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters. Five mice were used for each treatment group. Points, mean; bars, SE. The data was analyzed using Student's t test. Arrow, day of treatment. Two independent experiments are shown. P < 0.05 from day 5 in experiment 1; P < 0.05 from day 7 in experiment 2. B–E, histological study using the TdT-mediated biotin-dUTP labeling technique. Tumors were harvested 5 days after the beginning of treatment and immediately embedded into OCT compound. Frozen tissues were cut in a cryostat at 5-μm thicknesses. The sections were treated with 1 μg/ml proteinase K and stained as described in the legend to Fig. 3. Pictured are H358 tumors treated with (8) CDDP alone, (C) Ad-p53 alone, or (D and E) Ad-p53 in the combination with CDDP. Bars, 0.5 mm. All animal care was in accordance with the UT M. D. Anderson Institutional Animal Care and Use Committee.

chemotherapeutic drugs can occur within 6 h after DNA-damaging stimuli (3, 20), although increased p53 DNA binding activity can be reversed over the course of 4 h if the stimulus is removed (21).

In our model, the expression of the wt-p53 gene is driven independently by the cytomegalovirus promoter contained in an Ad-p53 vector. Therefore, a high level of p53 expression can be maintained even after cessation of drug exposure. The expression of wt-p53 protein by Ad-p53 peaks at postinfection day 3 (14-fold greater than endogenous wild-type) and decreases to a low level by day 9 (22). This suggests that a transiently high level of wt-p53 expression is sufficient to initiate the cytotoxic program in the cancer cell.

Our data indicate that p53 has an important role as a determinant of chemosensitivity in human lung cancer cells. A variety of treatment protocols, including surgery, chemotherapy, and radiotherapy, have been tried for human NSCLC, but the long-term survival rate remains unsatisfactory. The combination therapy we present here might be effective as an adjuvant treatment to prevent local recurrence following primary tumor resection or as a treatment that could be given by intrallesional injections in drug-resistant primary, metastatic, or locally recurrent lung cancer. Protocols are being developed to explore these clinical applications.

References

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