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

Toshiyoshi Fujiwara, Elizabeth A. Grimm, Tapas Mukhopadhyay, Wei-Wei Zhang, Laurie B. Owen-Schaub, and Jack A. Roth

Section of Thoracic Molecular Oncology, Department of Thoracic and Cardiovascular Surgery [T. F., T. M., W. W. Z., J. A. R.], and Departments of Tumor Biology [E. A. G.], Surgical Oncology [E. A. G.], and Immunology [L. B. O.-S.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

Recombinant adenovirus-mediated transfer of the wild-type p53 gene into monolayer cultures or multicellular tumor spheroids of human nonsmall 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 chemotherapeutic 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 16 h 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 DNA cross-linking agents 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 Xbal and ClaI sites of pXCI. 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-isooamyl alcohol (24:1) and then precipitated in ethanol. Samples were subjected to electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. TdT-mediated dUTP nick end labeling was performed according to a procedure reported previously (12). Monolayer cells were treated with 0.01% Nnionidet P-40. The slides were immersed in TdT buffer [30 mM Tris-HCI (pH 7.2)-140 mM sodium cacodylate-1 mM cobalt chloride] and incubated with biotinylated dUTP (Boehringer Mannheim, Indianapolis, IN) and TdT at 37°C for 45 min. The slides were covered with 2% bovine serum albumin for 10 min and incubated with avidin-biotin complex (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA) for 30 min. The colorimetric detection was performed by using diaminobenzidine.

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

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2 To whom requests for reprints should be addressed, at Department of Thoracic Surgery, UT M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

3 The abbreviations used are: wt-p53, wild-type p53; NSCLC, non-small cell lung cancer; CDDP, cisplatin; cDNA, complementary DNA; Ad-p53, recombinant adenovirus vector that contains the cDNA that encodes human wt-p53; Ad-Luc, recombinant adenovirus vector that contains the cDNA that encodes luciferase; TdT, terminal deoxynucleotidyl transferase.

4 T. Fujiwara, E. A. Grimm, T. Mukhopadhyay, and J. A. Roth, unpublished data.
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Introduction of wt-p53 is known to induce apoptosis in some types of tumor cell lines with deleted or mutated p53 (13–15). However, overexpression of wt-p53 alone could not promote DNA fragmentation in our p53-negative H358 cell line (Fig. 2), although their growth was suppressed by Ad-p53 (Fig. 1). This is compatible with our previous studies showing that stable H358 clones could be obtained after retrovirus-mediated wt-p53 transfer and that the clones grew more slowly than parental cells (16).

The potential therapeutic efficacy of the combination of Ad-p53 and CDDP was evaluated in terms of the relative change in volume of H358 spheroids. The multicellular tumor spheroid model exhibits in vitro a histological structure similar to that of primary tumors and micrometastases. Treatment with CDDP caused a reduction of relative volume in Ad-p53-infected H358 spheroids but had no significant effect on parental or Ad-Luc-infected spheroids (Fig. 3A). In situ TdT-mediated dUTP labeling showed many apoptotic cells in Ad-p53-infected spheroids whereas no apoptotic cells were seen on spheroids not infected with Ad-p53 (Fig. 3, B–E). We reported previously that retroviral-mediated wt-p53 expression inhibited growth of H322a spheroids induced by transforming growth factor α (TGF-α) (17). The retroviral vector could not infect H358 spheroids, however, because cells in these spheroids did 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 × 10⁴ cells/well) were seeded in duplicate on a 24-well plate. After 24 h, 100 µl of medium, Ad-Luc viral stock (10⁶ PFU/ml), or Ad-p53 viral stock (10⁶ 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 \( a^2 \times b_1^2 \times b_2 \), 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-\( iK \)) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the anti viral agent ganciclovir (19). The HS-\( iK \) 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
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 intratumoral injections in drug-resistant primary, metastatic, or locally recurrent lung cancer. Protocols are being developed to explore these clinical applications.

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