Growth Inhibition of Human Cervical Cancer Cells with the Recombinant Adenovirus p53 in Vitro\(^{1}\)

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Received May 1, 1995

Human papillomavirus (HPV) has been identified in the majority of invasive cancers of the uterine cervix sampled and has been found to contribute in a significant way to the genesis of human cervical cancer. HPV has two transforming genes that encode the oncoproteins E6 and E7. E6 can form complexes with p53 and promote p53 degradation. We introduced wild-type p53 into a cervical cancer cell line via a recombinant adenoviral vector, Ad5CMV-p53. Human cervical cancer cell line HeLa, which has HPV type 18 and wild-type p53, was used in this study. Cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum. Ad5CMV-p53 was created by inserting the cytomegalovirus promoter, wild-type p53 cDNA, and SV40 polyadenylation signal in a minigene cassette into the E1-deleted region of the modified Ad5 adenovirus. The transduction efficiency was 100% when a dose ensuring a multiplicity of infection of 100 or greater was used. The p53 protein was detected in Ad5CMV-p53-infected cells by immunohistochemical and Western blot analyses. The growth of the Ad5CMV-p53-infected cells was greatly suppressed as detected by both cell count and \(^{3}\)Hthymidine incorporation assay. These data suggest that transfection of HPV-positive cervical cancer cells with a wild-type p53 gene in a form such as Ad5CMV-p53 is a potential novel therapy for cervical cancer.

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\(^1\) This study was partially supported by grants from the National Cancer Institutes and the National Institute of Health (RO1 CA45187) (J.A.R.); by gifts to the Division of Surgery from Tenneco and Exxon for the Core Laboratory Facility; by the M. D. Anderson Cancer Center Support Core Grant (CA16672); and by a grant from the Mathers Foundation, the University Cancer Foundation, and the Business and Professional Women’s Fund. 

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INTRODUCTION

Cervical cancer is the second most common malignancy in women worldwide, accounting for 15% of all cancers diagnosed in women [1]. In 1989, 500,000 cases were expected worldwide and an overall 5-year survival rate is 40% [1]. Despite aggressive screening with the Papanicolaou smear, this cancer remains an important health problem for women. The most important risk factor is human papillomavirus (HPV) infection, which increases the relative risk of developing cervical intraepithelial neoplasia and cervical cancer by 11- to 60-fold [1].

HPV types 16 and 18 have been identified in anywhere from 50–90% of cervical cancers [2–4]. The E6 and E7 genes of HPV 16 and 18 are frequently coexpressed and are the most abundant viral transcripts in biopsies from HPV-positive cervical cancers [5, 6]. Virus-encoded oncoproteins are important cellular regulatory proteins by which viruses can transform cells. The E6 and E7 proteins of HPV 16 and 18 have been demonstrated to be necessary and sufficient for malignant transformation of mammalian cells [7, 8]. Furthermore, the continued expression of the E6 and E7 region of the viral genome appears to be necessary for the maintenance of the malignant phenotype [9]. E7 forms complexes with the retinoblastoma protein (pRB) and E6 forms complexes with the p53 protein.

E7 shares functional and structural features with the adenovirus E1A proteins. The E7 proteins of the “high-risk” HPVs for the pRB have an approximately 10-fold higher affinity than do E7 proteins of the “low-risk” HPVs; this is thought to be due to the inclusion of the pRB-binding domain in the amino acid terminal sequences of these proteins [9]. The E6 oncoprotein shares functional and structural features with SV40 large T and adenovirus 5 E1B. In vitro, E6 promotes degradation of p53 via the ubiquitin-dependent protease system. This selective degradation of negative regulatory proteins provides a novel mechanism for dominant acting oncoproteins [10–12]. Howley and others have demonstrated that the inactivation of RB and p53 are important steps in cervical carcinogenesis [13]. HPV-positive cell lines expressed normal pRB and low levels of wild-type p53 [13].
The wild-type p53 gene may have important roles not only in cell cycle but in apoptosis.

Growth of human non-small cell lung cancer cells and squamous cell carcinomas of the head and neck was significantly inhibited in vitro and in vivo by the introduction of wild-type p53 via a recombinant adenoviral vector, Ad5CMV-p53 [14, 15]. The p53 adenovirus was directly injected into non-small cell lung cancer cells with a homozygous deletion of p53; subcutaneous implantation of these cells in nu/nu mice, followed by intraperitoneal administration of cisplatin, induced massive apoptotic destruction of the tumors [16]. HPV-immortalized human cervical epithelial cell lines cannot tolerate large amounts of exogenous wild-type p53 transfected by Lipofectin [17]. However, there are no reports on the efficacy of the p53 adenoviral vector on cervical cancer cells.

We introduced the wild-type p53 gene into cervical carcinoma cells via the recombinant adenoviral vector, Ad5CMV-p53, to determine its effects on growth of the HPV-18-positive human cervical cancer HeLa cell line.

**MATERIALS AND METHODS**

**Cell Line**

Human cervical carcinoma cell line HeLa was obtained from the American Type Culture Collection (Bethesda, MD). HeLa contains either HPV-18 or HPV-related sequences and expresses p53 mRNA which has a wild-type sequence [18]. Cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum.

**Recombinant Adenovirus and Infection**

The recombinant p53 adenovirus (Ad5CMV-p53) contains the cytomegalovirus promoter, wild-type p53 cDNA, and SV40 polyadenylation signal in a minigene cassette which is inserted into the E1-deleted region of modified adenovirus Ad5 [14]. The adenoviral vector containing the same cassette but without p53 cDNA was used as control (Ad5CMV-poly A). The adenovirus containing the β-galactosidase (β-gal) gene, Ad5CMV-LacZ, was used to determine transduction efficiencies. Viral stocks were propagated in 293 cells, which are derived from primary embryonal kidney cells transformed by introducing sheared fragments of Ad5 DNA. This cell line contains E1A and is thus highly permissive of the replication of the E1 replication-deficient adenovirus. Cells were harvested 36–40 hr after infection, pelleted, resuspended in phosphate-buffered saline, and lysed; cell debris was removed by subjecting the cells to CsCl gradient purification. Concentrated virus was dialyzed and stored in aliquots at −80°C. Infection was carried out by adding the virus to high-glucose Dulbecco’s minimal essential medium and to the cell monolayers. The cells were incubated at 37°C for 60 min with constant agitation. Medium was added, and the cells were incubated at 37°C for the desired length of time.

**Northern Blot Analysis**

Total RNA was isolated by the RNAzol B method (TEL-TEST Inc., Friendswood, TX). Northern analysis was performed on 20 µg of total RNA. The membrane was hybridized with a p53 cDNA probe labeled by the random primer method in 5 × SSC-5 × Denhardt solution–0.5% SDS–denatured salmon sperm DNA (20 µg/ml).

**Western Blot Analysis**

Total cell lysates were prepared by lysing monolayer cells in dishes with SDS–PAGE sample buffer. Five micrograms of protein samples was subjected to 10% SDS–polyacrylamide gel electrophoresis and transferred to Hybond-ECL membrane (Amersham Corp., Arlington Heights, IL). The membrane was blocked with 1% nonfat dry milk and 0.1% Tween 20 (Sigma Chemical Co., St. Louis, MO) and probed with the primary antibodies, mouse anti-human p53 monoclonal antibody DO7 (DAKO, Carpinteria, CA), and mouse anti-human actin monoclonal antibody (Amersham), and the secondary antibody, horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham).

**Immunohistochemical Analysis**

The infected cell monolayers were fixed with 3.8% formalin and treated with 3% H2O2 in methanol for 5 min. Immunohistochemical staining was performed by using the Vectastain Elite kit (Vector Labs, Burlingame, CA). The primary antibody was the anti-p53 antibody DO7 (DAKO, Carpinteria, CA), and mouse anti-human actin monoclonal antibody (Amersham), and the secondary antibody, horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham).

**Cell Count Assay**

Cells were plated at a density of 5 × 10⁴/ml/well in 12-well plates in triplicate. RPMI supplemented with 10% heat-inactivated fetal bovine serum was used as growth medium. Cells were infected with either Ad5CMV-p53 or the Ad5CMV-poly A control. Cells were harvested and counted at each point of the dose and the date. Cell viability was determined by trypan blue exclusion.

**[^H]Thymidine Incorporation Assay**

Cells were also cultured at 2000 cells/well in 96-well flat-bottomed plates. Cells were infected with either Ad5CMV-p53 or Ad5CMV-poly A and cultured in RPMI medium containing 10% fetal bovine serum for 5 days. Each well was pulse-treated with 1 µCi [^H]thymidine (sp
FIG. 1. Transduction efficiency in the HeLa cell line. A recombinant β-gal adenovirus, Ad5CMV-LacZ, was used to infect the cells at different m.o.i.s ranging from 0.2 to 200. The percentages of β-gal-positive cells were obtained from scoring 500 cells each on replicate dishes.

RESULTS

The conditions for optimal adenoviral transduction were determined by infecting cells with an adenovirus, Ad5CMV-LacZ, that expresses the β-gal gene. The transduction efficiency was assessed by counting the percentage of blue cells after X-gal staining. The percentage of β-gal-positive cells was obtained by scoring 500 cells each on replicate dishes. There appeared to be a linear relationship between the number of infected cells and the number of adenovirus particles used in the infection. Cells inoculated with a single dose of Ad5CMV-LacZ at a multiplicity of infection (m.o.i.) of 100 exhibited 100% blue cells (Fig. 1). The transduction efficiency of this vector in HeLa cells was similar to those in other cell lines examined previously, HepG2, LM2, and human non-small cell lung cancer cell lines [14].

The recombinant wild-type p53 adenovirus, Ad5CMV-p53, was used to infect the cervical cancer cell line HeLa. Twenty-four hours after infection, total RNA was isolated, and Northern blot analysis was performed. The levels of the 2.8-kilobase endogenous p53 mRNA detected in the samples isolated from mock-infected cells and from the cells infected with a control vector Ad5CMV-poly A were similar (Fig. 2A). The level of exogenous 1.9-kilobase p53 mRNA was increased in the cells infected with Ad5CMV-p53 (Fig. 2A), indicating that the exogenous p53 cDNA was successfully transduced into cells and efficiently transcribed. The level of endogenous 2.8-kb p53 mRNA was also increased in the cell infected with Ad5CMV-p53. Western blot analysis was performed to compare the amount of p53 protein produced. A p53 band, recognized by the monospecific anti-p53 antibody DO7, was observed in cellular extracts isolated from infected cells. Samples isolated from mock-infected (medium only) and Ad5CMV-poly A-infected cells did not exhibit the p53 protein (Fig. 2B). Immunohistochemical analysis of cells infected with Ad5CMV-p53 revealed the characteristic nuclear staining of the p53 protein (Fig. 3), whereas mock-infected cells failed to show the p53 protein.

The growth of the Ad5CMV-p53-infected cells was

FIG. 2. (A) Northern blot analysis. (a) Total RNA was isolated to detect expression of exogenous p53 mRNA 24 hr after Ad5CMV-p53 infection. (b) The ethidium bromide-stained gel scanned at the 18S and 28S rRNA band served as a standard for loading. Left lane is mock infection, middle lane is Ad5CMV-poly A infection, and right lane is Ad5CMV-p53 infection. (B) Western blot analysis. Cellular extracts isolated from cells 24 hr after infection were subjected to 10% SDS–polyacrylamide gel electrophoresis. Left lane is mock infection, middle lane is Ad5CMV-poly A infection, and right lane is Ad5CMV-p53 infection.

FIG. 3. Immunohistochemical staining of HeLa cells 24 hr after infection of Ad5CMV-p53 at 100 m.o.i.
FIG. 4. Inhibition of the growth of HeLa cells by cell count assay. At each indicated point, cells in three wells on each well plate were trypsinized and counted. The means of cell counts for triplicate wells 6 days after infection were plotted. Bars indicate SD.

greatly suppressed when estimated by cell count and \[^{3}H\]-thymidine incorporation assay (Figs. 4–6). Cell growth assays were reproducible in five repeated experiments. Twenty-four hours after infection, an apparent morphological change occurred, with portions of the cell population rounding up and their outer membranes forming blebs. These are part of a series of histologically predictable events that suggest programmed cell death. Cells infected with the con-
Control adenovirus, Ad5CMV-poly A, demonstrated normal growth characteristics with no histomorphological abnormalities.

**DISCUSSION**

HPV has been identified in the majority of invasive cancers of the uterine cervix and has been found to contribute in a significant way to the genesis of human cervical cancer. Inactivation of p53 gene by allelic loss or by point mutation is infrequent in primary cervical cancer and the overall incidence in these cancers of point mutations in the p53 gene is 1–6% [18]. HPV has two transforming genes that encode the oncoproteins E6 and E7. E6 can form complexes with p53 and promote p53 degradation. HPV-positive cell lines express low levels of wild-type p53 [13, 19]. Since the wild-type p53 gene is believed to be involved primarily in delivering antiproliferative signals that may be capable of antagonizing the growth-stimulatory signals propagated by oncoprotein products, the potential molecular therapeutic effect of this gene in cervical cancer deserves attention.

The rapid advancement in the field of gene therapy, including the creation of adenoviral vectors, has created an opportunity for progress toward novel gene therapy of cervical cancer. Because of their natural tropism for epithelial cells, adenoviruses may be uniquely suitable for the transient delivery of genes to cancers in epithelial tissues. The recombinant, replication-defective adenoviruses that have been developed for gene therapy are missing the entire E1A and part of the E1B regions and are, therefore, capable of propagating only in cells that can provide the E1 proteins in trans, such as the 293 cell line. In the past few years, recombinant adenoviruses have been extensively investigated as vectors for in vivo gene therapy. The high transfer efficiency of adenoviral vectors over a broad range of hosts both in vitro and in vivo make them attractive vehicles for molecular therapy.

The recent creation of a recombinant wild-type p53 adenoviral vector (Ad5CMV-p53) provided us with an excellent candidate for investigation of the biological effects of the wild-type p53 gene product on the cervical cancer cell line HeLa, in which the wild-type p53 gene is inactivated by HPV-18 infection. A β-gal recombinant adenovirus allowed us to establish the gene transfer efficiency in cervical cancer cells. At more than 100 m.o.i., all cervical cancer cells were positive for X-gal staining. There appeared to be a linear correlation between the number of cells expressing the gene and the amount of viral particles used in the experiment. This result coincided with the efficiency obtained in cells infected with Ad5CMV-p53, which was assessed by immunostaining using a monoclonal anti-p53 antibody. The transduction efficiency we observed was almost the same as these achieved in other cell lines tested, including HepG2, LM2, and the human non-small cell lung cancer cell lines.

The p53 gene is wild-type in HeLa cells. The p53 mRNA is detected in untransfected HeLa cells, but p53 protein was barely detected in untransfected cells. Western blot and immunohistochemical analyses demonstrated that production of p53 protein was significantly higher in Ad5CMV-p53-
infected cells than in control cells, suggesting that the exogenous p53 mRNA was efficiently translated. Time-course protein expression studies in human non-small cell lung cancer cells have shown protein expression to peak 3 days after infection and progressively decline to levels still detectable by Western blotting on Day 15, but nondetectable levels 3 weeks after infection [14]. In most systems of adenoviruses, expression is transient.

The cervical cancer cells transduced with the wild-type p53 gene exhibited significantly more inhibition of growth in vitro then mock-infected and control adenovirus-infected cells, clearly illustrating that these results were not mediated by the virus itself. The mechanism by which wild-type p53 protein inhibits growth in vitro may be related to arrest of the cell cycle at G1, apoptosis, or induction of DNA fragmentation. satisfactory. A treatment strategy based on restoration of p53 function requires further investigation including DNA ladder, chemotherapy, and radiotherapy, have been tried for human cervical cancer cell lines in vitro and in vivo. However, the mechanism of growth suppression and cell death induced by Ad5CMV-p53 requires further investigation including DNA ladder, morphological demonstration of nuclear condensation, and DNA fragmentation.

Adenoviral vectors have many advantages over other viral vector systems and other in vitro techniques for introducing DNA into eukaryotic cells. Human adenoviruses are double-stranded DNA viruses with an average genome size of approximately 36 kb, and thus have the potential to carry large or multiple foreign genes. Adenoviruses are easy to grow and manipulate, and they have a broad range of hosts in vitro and in vivo. Because the life cycle of adenovirus does not require integration into the host genome, the foreign genes delivered by adenoviral vectors are expressed episomally and therefore have low genotoxicity to host cells. Adenoviruses appear to be linked only to mild diseases, since there is no known association of human malignancies with adenoviral infection. Moreover, no side effects have been reported following vaccination with wild-type adenoviruses, demonstrating their safety and therapeutic potential as in vivo gene transfer vectors [23]. Recombinant adenoviruses have been successfully administered to a variety of tissues through different routes, including intratracheal instillation [24], muscle injection [25], peripheral intravenous injection [26], and stereotaxic inoculation to the brain [27].

Several studies with different gene products have revealed that following infection in a wide variety of target tissues, only transient expression is observed [28–30]. Since the cells infected with the recombinant adenovirus are rapidly eliminated, it is likely that the host immune system plays a major role in preventing sustained expression of the foreign genes. Recent results where a temperature-sensitive E2A gene was used to construct recombinant adenovirus, suggests that cytotoxic T lymphocyte response to viral antigens can be reduced at nonpermissive temperature [31]. Thus it is hopeful that a second generation of adenoviral vectors where E1A, E2A, and E4 gene have been deleted or rendered ineffective will be able to reduced the cell-mediated immunity problem.

Gene replacement with the wild-type p53 gene has reversed features of the malignant phenotype, including soft agar colony formation, rapid cell proliferation, and tumorigenicity, in human tumor cells with homozygous p53 deletions or mutant p53 genes [14, 16, 21]. Human cervical cancer cells have a functional genotype equivalent to cells with an inactivated p53 gene product due to the complexing of the E6 protein with the p53 protein. Restoration of p53 function in these cells is effective in reducing proliferation. The adenoviral vector is capable of mediating high levels of p53 expression which apparently are sufficient to overcome the capabilities of the endogenous E6 to bind the protein.

Our data indicate that p53 has an important role as a determinant of malignant cell growth in human cervical cancer cells. A variety of treatment protocols, including surgery, chemotherapy, and radiotherapy, have been tried for human cervical cancer, but the long-term survival rate remains unsatisfactory. A treatment strategy based on restoration of tumor suppressor gene function might be effective as an adjuvant treatment to prevent local recurrence following primary tumor resection or as a treatment that could be given by intralesional injections in drug-resistant primary, metastatic, or locally recurrent cancers. Protocols are being developed to explore these clinical applications.

ACKNOWLEDGMENTS

We sincerely thank Ms. Ronetta Fairchild and Ms. Carol Torrence for their help in preparing the manuscript. We also sincerely thank Ms. Yoko Akimaru for her help in preparing the immunohistochemical slides.

REFERENCES

ADENOVIRAL VECTOR INHIBITS CERVICAL CANCER CELLS IN VITRO


