Advances in Brief

Cell Cycle Arrest and Inhibition of Tumor Cell Proliferation by the p16\(^{\text{INK4}}\) Gene Mediated by an Adenovirus Vector\(^1\)

Xiaomei Jin, Dao Nguyen, Wei-Wei Zhang, \(^2\) Athanassios P. Kyrkiris, and Jack A. Roth\(^3\)

Section of Thoracic Molecular Oncology, Departments of Thoracic and Cardiovascular Surgery [X. J., D. N., W-W. Z. J. A. R.], Neuro-Oncology [A. P. K.], and Tumor Biology [J. A. R.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

The p16\(^{\text{INK4}}\) (MTS1) gene has many features of a tumor suppressor gene. It maps to 9p21, a region of frequent loss of heterozygosity in a variety of tumor types. It encodes an inhibitor of cyclin-dependent kinase 4, and its homozygous deletion is common in tumor-derived cell lines. To examine its tumor suppressive function and its potential in cancer gene replacement therapy, wild-type p16\(^{\text{INK4}}\) was expressed in an adenovirus-derived gene delivery system and introduced into lung cancer cell lines that do not express p16\(^{\text{INK4}}\). Expression of the introduced p16\(^{\text{INK4}}\) blocked tumor cell entry into S phase of the cell cycle and inhibited tumor proliferation both in vitro and in vivo. These observations strongly support that p16\(^{\text{INK4}}\) is a tumor suppressor gene and is a candidate for cancer gene replacement therapy.

Introduction

The major transitions of the eukaryotic cell cycle are triggered by the CDKs.\(^4\) CDK4 regulates progression through the G\(_1\) phase of the cell cycle. In normal cells, the retinoblastoma tumor suppressor protein (Rb) regulates cell proliferation by binding and sequestering transcription factors essential for progression to S phase. These transcription factors are released at late G\(_1\) by phosphorylation of Rb, thereby allowing cells to enter S phase. The main function of the CDK4-cyclin D complexes may be to phosphorylate Rb at late G\(_1\). The activity of CDK4 is controlled by an activating subunit, D-type cyclin, and by an inhibitory subunit, the p16\(^{\text{INK4}}\) protein, which is encoded by the p16\(^{\text{INK4}}\) gene. The p16\(^{\text{INK4}}\) gene has been biochemically characterized as a protein that specifically binds to and inhibits CDK4, and thus it may regulate Rb phosphorylation (1, 2). The p16\(^{\text{INK4}}\) protein belongs to a newly described class of CDK-inhibitory proteins that also includes p15\(^{\text{INK4B}}\), p21\(^{\text{WAF1}}\), and p27\(^{\text{KIP1}}\). The p16\(^{\text{INK4}}\) gene maps to 9p21, a chromosome region frequently deleted in many tumor types. Homozygous deletions and mutations of the p16\(^{\text{INK4}}\) gene are frequent in human tumor cell lines. This evidence suggests that the p16\(^{\text{INK4}}\) gene is a tumor suppressor gene. This interpretation has been challenged by the observation that the frequency of p16\(^{\text{INK4}}\) gene alterations is much lower in primary uncultured tumors than in cultured cell lines (3–12). To examine directly the suppressive function of the p16\(^{\text{INK4}}\) gene and its potential in cancer gene therapy, we inserted the p16\(^{\text{INK4}}\) gene in a recombinant adenovirus and delivered it into lung cancer cell lines carrying homozygous deletions of this gene. The expression of the introduced p16\(^{\text{INK4}}\) gene arrested tumor cell growth at G\(_1\) and led to a pronounced inhibition of tumor cell growth both in vitro and in vivo.

Materials and Methods

Cell Lines. Cell line 293 was maintained in Eagle’s modified essential medium supplemented with 10% heat-inactivated horse serum. Human NSCLC cell lines H226Br, H322, and H460 were gifts from Drs. J. D. Minna and A. F. Gazdar (University of Texas Southwestern Medical Center at Dallas, Dallas, TX) and were grown in RPMI medium containing 5% fetal bovine serum. Human normal breast cell line HBL100 was a gift from Dr. Dihua Yu (M. D. Anderson Cancer Center) and was grown in F12 medium supplemented with 10% fetal bovine serum.

p16\(^{\text{INK4}}\) cDNA Subcloning. The original p16\(^{\text{INK4}}\) cDNA was amplified from the total RNA of normal human lymphocytes by reverse transcription–PCR with the use of the primers 5’ATGAGCCCTGTCGCTGACG3’ and 5’CCTGTAGGACCTTCGGTGACTGG3’. The PCR product was subcloned in pCR vector (Invitrogen, San Diego, CA) and verified by double-stranded DNA sequencing. Because of a correction of the p16\(^{\text{INK4}}\) cDNA sequence in Gene Bank, an additional sequence of 42 bp was added later to the 5’ end of the cloned p16\(^{\text{INK4}}\) DNA by two PCR steps. The first PCR step used primer A (5’GATCCGGCGGCGGGGAGCAGCATGG AGCCTTCGGCTG ACTGG3’) and primer C (5’GCCTCTCCTGTTGCTCTTCA3’). The second PCR step used primer B (5’GGGGCGGGAGACGTAGGACGCGGGGGGGGAGG3’) and primer C. The final wild-type p16\(^{\text{INK4}}\) cDNA sequence in the pCR vector (pCR-p16) was again verified by double-stranded DNA sequencing.

pAd-p16 Construction. The shuttle vector pEC53 (13) was digested by restriction enzymes HindIII and HpaI. The vector backbone was separated from p53 cDNA by running the digested DNA through 1% agarose gel and was purified from the gel. p16\(^{\text{INK4}}\) cDNA was excised from pCR-p16 and ligated to the purified shuttle vector backbone. The final product, pAd-p16, carries the p16\(^{\text{INK4}}\) expression cassette that contains the human CMV promoter (14) wild-type p16\(^{\text{INK4}}\) cDNA and SV40 early polyadenylation signal.

Generation of Recombinant p16\(^{\text{INK4}}\) Adenovirus. The recombinant Ad5CMV-lacZ adenovirus DNA was digested with restriction enzymes XbaI and CdiI, and the 32-kb partial adenovirus DNA fragment was purified in a 0.3% agarose gel. This DNA fragment and pAd-p16 plasmid DNA were cotransfected into 293 cells by CaPO\(_4\)-mediated transfection. The transfected cells were maintained in medium until the onset of the cytopathic effect. The newly generated p16\(^{\text{INK4}}\) recombinant adenovirus (Ad-p16) was identified by PCR analysis of the DNA samples prepared from the cell culture supernatant. The recombinant adenovirus Ad5CMV-lacZ (a gift from Dr. Frank L. Graham, McMasters University, Hamilton, Ontario, Canada), which carries the lacZ gene of Escherichia coli, has a structure similar to that of Ad-p16 and was used as a control in these experiments.

Viral Stocks, Titers, and Infection. Individual clones of the Ad-p16 and Ad5CMV-lacZ viruses were obtained by plaque purification and were propagated in 293 cells according to the method of Graham and Prevec (15). The viral titers were determined by plaque assays. The cell lines were infected by the addition of the viral solutions to cell monolayers and incubation at room temperature for 30 min with brief agitation every 5 min. This was followed by the addition of fresh medium, and the infected cells were observed daily for cytopathic effect.

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2 Present address: Gene Therapy Unit, Baxter Healthcare Corporation, Baxter Technology Park, Route 120 and Wilson Road, Round Lake, IL 60073-0490.

3 To whom requests for reprints should be addressed, at M. D. Anderson Cancer Center, Department of Thoracic and Cardiovascular Surgery, Box 109, 1515 Holcombe Boulevard, Houston, TX 77030.

4 The abbreviations used are: NSCLC, non-small cell lung cancer; RT, reverse transcription; CDK, cyclin-dependent kinase; CDK4, cyclin-dependent kinase 4; CMV, cytomegalovirus; PFU, plaque-forming units.

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the addition of culture medium and the return of the infected cells to the 37°C incubator.

**Tumorigenicity Assays.** H460 cells were infected with Ad-p16 or Ad5CMV-lacZ at an multiplicity of infection of 50 PFU/cell. An equal number of cells were treated with medium only as a mock infection. Twenty-four h after infection, the treated cells were harvested and rinsed twice with PBS. For each treatment, 5 million (5 × 10⁶) cells in a volume of 0.1 ml of PBS were injected s.c. into the dorsal flank of BALB/c nu/nu mice (Harlan Sprague-Dawley Co., Houston, TX). The treated mice were examined weekly after injection. Tumor generation was evaluated at the end of a 3-week period. Tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of orthogonal diameters.

**Results and Discussion**

**Generation of the Ad-p16 Recombinant Virus.** One of the advantages of adenovirus as a gene transfer vector is that it has high infectivity in a wide range of host cells (16). An adenovirus-derived shuttle vector for human cancer gene therapy, pEC53, was constructed by us previously (13). The recombinant virus derived from this vector, Ad5CMV-p53, has an infectivity of 97–100% in several lung cancer cell lines (13), including H460, H322, and H226Br. In our study, the p53 gene in pEC53 was replaced by the full-length p16INK4 cDNA, and a SV40 early polyadenylation signal. A 32-kb partial fragment of adenovirus DNA generated by Xbal digestion of the DNA of a recombinant virus, Ad5CMV-lacZ, was cotransfected with pAd-p16 plasmid into 293 cells for homologous recombination. The recombinant viral product, Ad-p16, has a genomic structure similar to that of Ad5CMV-lacZ except that the lacZ gene is replaced by the p16INK4 gene. Because the E1 regions of the recombinant adenoviruses are substituted by the p16INK4 gene or the lacZ gene expression cassette, they can be propagated only in 293 cells that complement the E1 deletion. Ad5CMV-lacZ was used as a viral control for Ad-p16.

**Expression of Exogenous p16INK4 Protein in Human Lung Cancer Cells.** Three human NSCLC cell lines were chosen for this study: H460, H322, and H226Br. H460 carries a homozygous p16INK4 gene deletion (6), but the p53 gene (17) and the Rb gene (18) are wild type. H322 carries a homozygous p16INK4 gene deletion (9), a wild-type Rb gene (18), and a homozygous p53 mutation at codon 248 (19). H226Br carries the p16INK4 gene detected by Southern blot and PCR analysis (data not shown) that has not been sequenced but does not express p16INK4 at the protein level (Fig. 1A). It carries a homozygous p53 mutation at codon 254 (20) and expresses wild-type RB protein (18). A cultured normal human breast epithelial cell line, HBL100, expresses wild-type p16INK4, wild-type p53, and wild-type Rb genes⁵ and was used in this study as a normal cell line control. As shown in Fig. 1A, only cell line HBL100 expressed p16INK4 protein before viral infection. To obtain a high level of expression of exogenous p16INK4 protein, the human CMV promoter (14) was used to drive the expression of the p16INK4 gene. As shown in Fig. 1B, high levels of exogenous p16INK4 protein expression were achieved in the H460, H322, and H226Br cells after infection with Ad-p16. The level of p16INK4 protein in HBL100 was much higher after Ad-p16 infections (data not shown), indicating exogenous p16INK4 protein expression in this cell line. Subsequently, the effect(s) of this introduced p16INK4 protein on the tumor cell lines was examined in the following assays.

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⁵ M-C. Hung, personal communication.

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**Effect of p16INK4 Protein on Lung Cancer Cell Growth.** The NSCLC cell lines H460, H322, and H226Br and the normal breast cell line HBL100 were infected with Ad-p16 or Ad5CMV-lacZ at 50 PFU/cell. Triplicate sets of the infected and mock-infected cells were counted every day for 6 days, and the mean cell number for each day was calculated. As shown in Fig. 2, growth rates of the Ad-p16-infected H460, H322, and H226Br cells were inhibited by 94, 85, and 97%, respectively, compared with that of the Ad5CMV-lacZ-infected cells. However, the growth rate of HBL100 infected with Ad-p16 was inhibited by <3% compared with that of the Ad5CMV-lacZ-infected HBL100 cells. This suggested that introduction of the p16INK4 gene into these cell lines could specifically suppress cell proliferation by restoring p16INK4 expression. The growth rates of the Ad5CMV-lacZ-treated cells were lower than those of the mock-infected cells for all of the cell lines, indicating cytotoxicity caused by expressed viral proteins and the lacZ gene. This virus-related cytotoxicity was increased when a higher multiplicity of infection was used (data not shown). The cell lines had differing sensitivities to this effect.

**Cell Cycle Arrest Mediated by Ad-p16.** It is known that the p16INK4 protein can inhibit the activity of CDK4, thereby blocking the entry of the proliferating cells from G₁ to S phase (1, 2). To examine the mechanism of the growth rate inhibition mediated by Ad-p16, H460, H322, H226Br, and HBL100 cells were infected as described...
in the growth rate assay and harvested 24 h after infection for cell cycle analysis by flow cytometry. As shown in Table 1, Ad-p16-mediated expression of the p16INK4 protein significantly increased the numbers of cells in G1 and decreased the number of cells in S and G2 + M phases in the p16INK4-deleted tumor cell lines, suggesting the induction of G1 arrest. In contrast, no G1 arrest was observed in the p16INK4 protein-positive normal breast cell line HBL100. These results suggest that the p16INK4 protein suppresses the growth of the tumor cells by mediating G1 arrest in cell lines that do not express p16INK4.

Inhibition of Tumorigenicity Mediated by Ad-p16. To determine whether the Ad-p16 virus can inhibit tumorigenicity of human NSCLC cells, BALB/c nu/nu mice were given s.c. injections with H460 cells to induce tumor formation. Each mouse received one injection of 5 × 10⁶ cells that had been infected with either Ad-p16 or Ad5CMV-lacZ at 50 PFU/cell for 24 h. H460 cells treated with medium alone were used as mock-infected controls. Each treatment was given to four mice. The mice were observed, and when tumors appeared they were measured for a 3-week period. Two independent experiments were done to confirm reproducibility, and the data from both experiments are summarized in Table 2. Ad-p16-treated cells significantly suppressed tumor growth in vivo. One-hundred % of the mice that received medium treated cells and 87.5% of mice that received Ad5/CMV-lacZ-treated cells developed tumors. On the other hand, only 50% of mice in both experiments that received Ad-p16-treated cells developed tumors, and the mean volume was only 11% of that in Ad5/CMV-lacZ virus-treated mice and 6% of that in the medium-treated mice (P < 0.001 by two sided Student’s t test). Thus, the tumorigenicity of the lung cancer cells was inhibited by prior treatment with Ad-p16, indicating that the p16INK4 protein may have therapeutic efficacy.

The therapeutic potential for Ad-p16 was examined in a BALB/c nu/nu mouse model. s.c. tumor nodules arose 20 days after injecting mice s.c. with 5 × 10⁶ H460 cells. The resulting tumors were directly

<table>
<thead>
<tr>
<th>Cell types and infected viruses</th>
<th>% of cells in*</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>HBL100/Ad-p16</td>
<td>36</td>
<td>42</td>
</tr>
<tr>
<td>HBL100/Ad5CMV-lacZ</td>
<td>31</td>
<td>44</td>
</tr>
<tr>
<td>HBL100/medium</td>
<td>31</td>
<td>42</td>
</tr>
<tr>
<td>H460/Ad-p16</td>
<td>88</td>
<td>9</td>
</tr>
<tr>
<td>H460/Ad5CMV-lacZ</td>
<td>41</td>
<td>32</td>
</tr>
<tr>
<td>H460/medium</td>
<td>39</td>
<td>35</td>
</tr>
<tr>
<td>H322/Ad-p16</td>
<td>80</td>
<td>6</td>
</tr>
<tr>
<td>H322/Ad5CMV-lacZ</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td>H322/medium</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td>H226/Ad-p16</td>
<td>80</td>
<td>11</td>
</tr>
<tr>
<td>H226/Ad5CMV-lacZ</td>
<td>20</td>
<td>53</td>
</tr>
<tr>
<td>H226/medium</td>
<td>26</td>
<td>44</td>
</tr>
</tbody>
</table>

* Values are shown for a representative assay.

Table 2. Effect of p16INK4 on tumorigenicity of H460 cell line in nude mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of tumors/ no. of mice (%)</th>
<th>Mean volume (mm³ ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>Medium</td>
<td>4/4 (100)</td>
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<tr>
<td></td>
<td>Ad5CMV-LacZ</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td></td>
<td>Ad-p16</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>Medium</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td></td>
<td>Ad5CMV-LacZ</td>
<td>3/4 (75)</td>
</tr>
<tr>
<td></td>
<td>Ad-p16</td>
<td>4/4 (100)</td>
</tr>
</tbody>
</table>

Fig. 2. Cell growth curves of the Ad-p16 infected cell lines. The cells were inoculated at densities of 5 × 10⁴ in 60-mm culture dishes 24 h before infection and infected with Ad-p16 or Ad5CMV-lacZ at 50 PFU/cell. Culture medium alone was used for mock infection. Triplet cultures of each cell line for each treatment were counted daily from postinfection days 1 to 6. The curves are plotted from a representative assay of three experiments. Points, mean; bars, SD.
Ad5CMV-tocZ, or PBS (5 mice/group), s.c. tumor nodules were created by injecting with PBS (P < 0.001 by two-sided Student’s t test).

PFU of Ad-p16 or Ad5CMV-ΔacZ divided equally in 3 doses were injected on alternate cells. Adenovirus is an efficient expression vector that can be pro-

of the wild-type p16INK4 gene. The Ad-pl6 adenoviral vector medi-

ated high levels of wild-type p16INK4 expression in human NSCLC cells. Adenovirus is an efficient expression vector that can be pro-

duced in high titers (13). This vector system may therefore, have application in gene replacement strategies for cancer treatment.

References
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