Adenovirus-Mediated p14^{ARF} Gene Transfer in Human Mesothelioma Cells

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Background: The p14^{ARF} protein encoded by the INK4a/ARF locus promotes degradation of the MDM2 protein and thus prevents the MDM2-mediated inhibition of p53. Homozygous deletion of the INK4a/ARF locus is common in human mesothelioma and may result in the loss of p14^{ARF} and the inactivation of p53. We designed this study to evaluate the biologic and potential therapeutic roles of p14^{ARF} expression in mesothelioma cells. Methods and Results: We constructed Adp14, an adenoviral vector carrying human p14^{ARF} complementary DNA, and used it to transfect human mesothelioma cells to determine its effect on mesothelioma cells. Overexpression of p14^{ARF} led to increased amounts of p53 and the p21^{WAF} proteins and dephosphorylation of the retinoblastoma protein. The growth rate of mesothelioma cells was inhibited markedly by infection with Adp14 compared with mock infection or infection with a control adenovirus vector, AdCtrl. Overexpression of p14^{ARF} induced G_{1}-phase cell cycle arrest and apoptotic cell death. Cytotoxicity assays showed that Adp14 had a statistically significantly (P = .002) greater effect on colon cancer (HCT116) cell lines versus mock infection or infection with a control adenovirus vector. The transfection of p14^{ARF} into mesothelioma cells led to the overexpression of p14^{ARF}, which resulted in G_{1}-phase arrest and apoptotic cell death. These results suggest that this gene therapy-based approach may be of use in the treatment of mesothelioma. [J Natl Cancer Inst 2000;92:636–41]

Functional inactivation of the retinoblastoma protein (pRB) and p53 pathways appears to be a fundamental requirement for the development of most human cancers. Inactivation of these pathways disrupts regulation of the cell cycle and activates the apoptotic response. The INK4a/ARF locus (1) on human chromosome 9p21 (2,3) plays an important role in these two growth-control pathways by encoding two distinct proteins translated from alternatively spliced messenger RNAs. p16^{INK4a}, the cyclin-dependent kinase inhibitor, is specified by an RNA containing exons 1a, 2, and 3 (4), referred to as the α transcript. The alternative product, designated ARF for “alternative reading frame” (or p14^{ARF}, the human homologue), is specified by the β transcript, which contains exons 1b, 2, and 3 (5,6). The primary amino acid sequences of ARF and p16^{INK4a} are completely unrelated because the common exon 2 sequences are translated in different reading frames. p16^{INK4a} has been biochemically characterized as a protein that specifically binds to and inhibits cyclin-dependent kinase-4/6, and thus p16^{INK4a} regulates pRB phosphorylation and induces cell cycle arrest in G_{1} phase (4,7,8). ARF promotes MDM2 degradation and thus prevents the MDM2-mediated neutralization of p53 (9,10). Thus, a single mutational event at the INK4a/ARF locus, such as a homozygous deletion, has the potential to disrupt both pRB and p53 tumor suppressor pathways.

Normally, p53 is at a low concentration in a cell because of its relatively short half-life. Activation of p53 by the cellular stress induces the transcription of the MDM2 gene, and the MDM2 protein then binds to p53, which blocks the activities of p53 and promotes its degradation. These two proteins thus form an autoregulatory feedback loop in which p53 positively regulates MDM2 levels and MDM2 negatively regulates p53 levels and activity (11). Recent studies (9,10) have shown that the ARF protein can stabilize p53 by binding to and promoting the degradation of MDM2, providing a molecular mechanism for ARF function in cell cycle control and tumor suppression. Alterations in ARF function would result in overexpression of MDM2 and functional inactivation of p53.

Mesothelioma is an asbestos-related malignancy characterized by rapidly progressive and diffusely local growth, late metastases, and poor prognosis. Limited initial manifestations and lack of effective treatment make mesothelioma an attractive disease for gene-based therapies. Previous studies (12–14) showed that homozygous deletions of INK4a/ARF locus were the predominant events, with a frequency of more than 70% in mesothelioma. These deletions may result in the loss of p14^{ARF} and the functional inactivation of p53. Supporting the involvement of p14^{ARF}, we have not detected p14^{ARF} expression in 10 of 11 mesothelioma surgical samples (Yuan X, Yang, C-T, You L, Jablons DM; unpublished observations). In mesothelioma, unlike other adult malignancies, genetic alterations in p53 are reported to be rare (15,16). The replacement of the p14^{ARF} gene in mesothelioma cells may restore p53 function and thus result in cell cycle arrest and/or apoptotic cell death. In this study, we have introduced p14^{ARF} genes into human mesothelioma cells that lack p14^{ARF} genes by use of an adenoviral vector to determine whether we can restore p14^{ARF} function and to determine whether this gene therapy-based approach to treat an otherwise uniformly fatal disease is feasible.

Methods

Adenoviral vectors. The adenoviral vectors for gene expression studies were constructed by use of the AdEasy System (17). Briefly, the p14^{ARF} complementary DNA was first cloned into a shuttle vector, pAdTrack-CMV, that contained the p14^{ARF} protein into human mesothelioma cells.
cytomegalovirus promoters and a green fluorescent protein gene. The resultant plasmid was linearized by digesting with restriction endonuclease and was subsequently cotransformed into Escherichia coli BJ5183 cells with an adenoviral backbone plasmid, pAdEasy-1. Recombinants were selected for kanamycin resistance, and recombination was confirmed by restriction endonuclease analysis. The linearized recombinant plasmid was then transduced into 293 cells (E1-transformed human embryonic kidney cells). The p14ARF recombinant adenovirus Adp14 was generated and identified by polymerase chain reaction analysis of the DNA samples prepared from the cell culture supernatant. The control recombinant adenovirus AdCtrl, which carries a green fluorescent protein gene regulated by the cytomegalovirus promoter, was constructed with pAdTrack and pAdEasy-1 and used as a control in these experiments.

Cell lines and culture. H28 (CRL-5820), H513 (CRL-5830), H2052 (CRL-5915), and MSTO-211H (CRL-2081) mesothelioma cells were obtained from American Type Culture Collection (Manassas, VA). All were cultured in RPMI-1640 complete medium containing 10% fetal calf serum (FCS). HCT116 wild-type p53 (+/+) and p53-null (−/−) colon cancer cells were cultured in McCoy’s 5A complete medium supplemented with 10% FCS.

Immunoblotting. Approximately 1 × 10⁶ cells were plated in 10-cm dishes and incubated for 4 hours at 37 °C before transfection. They were then mock infected or infected with either AdCtrl or Adp14 (each at a multiplicity of infection of 50) and incubated for 72 hours. Samples were washed twice with phosphate-buffered saline (PBS), scraped off the plates, and lysed in cell lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]). Whole lysates were boiled, and the protein concentration was determined with the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (20–40 μg) were separated by SDS–polyacrylamide gel electrophoresis under reducing conditions in 4%–20% linear gradient polyacrylamide gels (Ready- Gel; Bio-Rad Laboratories). After the proteins were transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA), the membranes were blocked with 5% nonfat milk powder and 0.2% Tween 20 in Tris-buffered saline (TBS-T) overnight at 4 °C and then incubated with the primary antibody for 1 hour at room temperature. Membranes were then washed in TBS-T for three 5-minute periods. Primary antibodies for p14ARF (C-18), p16INK4A (C-20), p53 (DO-1), p21WAF1 [C-187, pRb (FP8), and actin (C-11) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse or donkey anti-goat antibodies were used as secondary antibodies (Santa Cruz Biotechnology). Proteins were visualized with chemiluminescence luminol reagents (Santa Cruz Biotechnology).

Measurement of cell viability. Cells (1 × 10⁶ H513 or 211H cells or 5 × 10⁴ H28 or H2052 cells) were seeded into six-well tissue culture plates, incubated for 4 hours at 37 °C, mock-infected or infected with Adp14 or AdCtrl (each at a multiplicity of infection of 50), and incubated for additional 3 or 7 days. Cells were collected after trypsinization and suspended in PBS. An equal volume of 0.4% trypan blue solution (Sigma Chemical Co., St. Louis, MO) was added to the cell suspension. Viable cells were then counted with a hemocytometer. All cell counts were done on triplicate samples.

Cytotoxicity assays. Cells were plated in triplicate wells of 96-well culture plates (500 cells/well) and incubated for 24 hours at 37 °C. AdCtrl and Adp14 were added at various concentrations and incubated for 7 days at 37 °C. A colorimetric assay was performed as described previously (18). Briefly, cells were fixed with trichloroacetic acid for 1 hour, washed five times with water, and air-dried. Cells were then stained for 30 minutes with 0.4% salidroside B (Sigma Chemical Co.) in 1% acetic acid and rinsed five times with 1% acetic acid to remove unbound dye. Bound dye was then solubilized with 10 mM unbuffered Tris-base (pH 10.5) for 5 minutes. Absorbance at 595 nm was measured with a kinetic microplate reader (Vmax; Molecular Devices Corp., Sunnyvale, CA) and was used as a measure of cell number. The IC₅₀ (dose that inhibited cell growth by 50%) was calculated by assuming that the survival rate of uninfected cells was 100%. The relative differences in IC₅₀ values were calculated by dividing the IC₅₀ of cells infected with Adp14 by the IC₅₀ of cells infected with AdCtrl.

Cell cycle analysis. Approximately 1 × 10⁶ cells were plated in 10-cm dishes, incubated for 4 hours at 37 °C, mock infected or infected with either AdCtrl or Adp14 (each at a multiplicity of infection of 50), and incubated for 48 hours. For time-course experiments, H513 cells were incubated up to 96 hours. The cells were collected after trypsinization, washed twice with 0.1% glucose in Mg²⁺/Ca²⁺-free PBS (sample buffer), and then fixed in ice-cold 70% ethanol overnight at 4 °C. Cells were then centrifuged 5 minutes at 3000 rpm at room temperature (Centra CL2 centrifuge with IEC 236 rotor; International Equipment Company, Needham Heights, MA). After decanting the 70% ethanol without disturbing the pellet, we stained the cells with propidium iodide (50 μg/mL) and ribonuclease A (10 U/mL) in sample buffer. Samples were analyzed by two-dimensional flow cytometry (FACScan; Becton Dickinson, Franklin Lake, NJ) to detect both green fluorescent protein and propidium iodide. The cell cycle distribution was determined by use of the cell cycle analysis software (ModFit LT; Verity Software House, Topsham, ME).

Annexin V apoptosis assay. Approximately 1 × 10⁶ H513 cells were plated in 10-cm dishes, incubated for 4 hours at 37 °C, mock infected or infected with AdCtrl or Adp14 (each at a multiplicity of infection of 50), and incubated for 96 hours. After each treatment, cells were collected after trypsinization, and apoptotic cells were assayed with an Annexin V-BIOTIN apoptosis detection kit (Oncogene, Cambridge, MA). Briefly, 5 × 10⁵ cells in 0.5 mL of PBS were incubated with 10 μL of media-binding reagent and 1.25 μL of Annexin V-BIOTIN for 15 minutes at room temperature in the dark. After the medium was removed, cells were gently suspended in 0.5 mL of ice-cold 1x binding buffer, and then 15 μL of phycoerythrin-conjugated streptavidin (15 μg/mL) was added. Finally, 10 μL of propidium iodide was added immediately before the analysis by flow cytometry. Early apoptotic cells with exposed phosphatidylserine but intact cell membranes bound Annexin V-BIOTIN-PE but excluded propidium iodide. Necrotic or apoptotic cells in terminal stages bound Annexin V-BIOTIN-PE and propidium iodide.

Statistical method. Results are expressed as means ± standard deviation. All statistical comparisons were made with a two-sided Student’s t test. A P value of less than .05 was considered to be statistically significant.

RESULTS

Expression of Exogenous p14ARF Protein in Human Mesothelioma Cells

We have generated an adenoviral vector, Adp14, that carries genes for p14ARF and the green fluorescent protein under control of the two separate cytomegalovirus promoters and a control vector, AdCtrl, that is similar to Adp14 except that it lacks the cytomegalovirus promoter-driven p14ARF gene. To investigate Adp14-mediated p14ARF expression in mesothelioma cells, we infected H28, H513, H2052, and MSTO-211H (211H) cells with Adp14 or AdCtrl. Forty-eight hours after transfection at a multiplicity of infection of 50, nearly 100% of the cells expressed the green fluorescent protein. As shown in Fig. 1, A, endogenous

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Expressions of p14ARF and p16INK4A in mesothelioma cell lines. A) In mesothelioma cell lines H28, H513, H2052, and 211H, endogenous p14ARF expression was absent, whereas substantial expression of p14ARF was detected by immunoblotting after infection with Adp14, a p14ARF-expressing adenovirus. B) Endogenous p16INK4A expression was not detected in all four mesothelioma cell lines. The C33A cervical cancer cell line was used as a positive control for the expressions of both p14ARF and p16INK4A. The cell lines used are shown below their corresponding lanes, the antibodies are shown to the left, and the infectious agents are above the lanes.
p14ARF expression was absent in all four mesothelioma cell lines. After Adp14 infection, however, expression of p14ARF was detected strongly by immunoblotting. These results show that all cell lines studied were infected with Adp14 and that infection resulted in a marked increase in p14ARF expression. To further investigate the genetic status of the INK4a/ARF locus, we also examined the expression of endogenous p16INK4a in these cell lines. We did not detect p16INK4a expression in any of the mesothelioma cells tested (Fig. 1, B).

Effects of p14ARF Overexpression on the p53–pRB Pathway

Because p14ARF stabilizes p53 by inhibiting MDM2, we looked for the effect of p14ARF overexpression on the expression of p53. Over a 3-day period at a multiplicity of infection of 50, Adp14-mediated expression of p14ARF resulted in a considerable increase in the amounts of both p53 and p21WAF proteins (Fig. 2, A). p21WAF, the universal cyclin-dependent kinase inhibitor, is a target for transcriptional activation by p53, inhibits cyclin-dependent kinase-mediated phosphorylation of pRB, and, therefore, leads to the arrest of cells at the G1-phase checkpoint (19). As shown in Fig. 2, B, overexpression of p14ARF led to dephosphorylation of pRB. As shown in lanes containing the mock-infected and AdCtrl-infected samples, two pRB bands were visible, representing the hyperphosphorylated and hypophosphorylated pRB. In the Adp14-infected samples, a dramatic decrease in the amount of the hyperphosphorylated pRB (upper bands) was observed, suggesting that overexpression of p14ARF led to pRB dephosphorylation.

Effect of Adp14-Mediated p14ARF Expression on Mesothelioma Cell Growth

The ability of Adp14 to inhibit the growth of human mesothelioma cells was examined. As shown in Fig. 3, A, cell growth of mock-, AdCtrl-, and Adp14-infected H513 cells was substantially suppressed by p14ARF expression. At 7 days, the numbers of viable cells in the Adp14-infected group were statistically significantly lower than those in mock- and AdCtrl-infected groups (P<.001 and P = .018, respectively; Fig. 3, A). Expression of p14ARF also resulted in markedly fewer viable cells at 72
hours and 7 days compared with AdCtrl-infected H28, H2052, or 211H cells (Fig. 3, B). The growth rates of the AdCtrl-infected cells were lower than the growth rates of the mock-infected cells for all four lines, which indicates the cytotoxicity from expressed viral proteins and overexpression of green fluorescent protein. The cell lines tested had different sensitivities to this virus-related toxicity.

**Adp14-Mediated p14ARF Expression, the G1/S-Phase Transition, and Apoptotic Cell Death in Human Mesothelioma Cells**

To examine the mechanism of the growth inhibition mediated by overexpression of p14ARF, we infected mesothelioma cells with Adp14 at a multiplicity of infection of 50 and harvested them 48 hours later for cell cycle analysis by flow cytometry. Adp14-mediated expression of p14ARF increased the numbers of cells in the G0/G1 phase and decreased those in the S and G2/M phases in all four cell lines, whereas infection with AdCtrl did not. Thus, p14ARF overexpression appears to inhibit cells at the G1/S-phase transition. A further time-course study was performed on H513 cells. After infection with Adp14 or AdCtrl (each at a multiplicity of infection of 50), cells were collected daily from days 1 to 4 after infection. As shown in Fig. 4, Adp14-mediated G1-phase arrest appeared at day 2. Furthermore, the numbers of cells in sub-G1 (<2N ploidy) populations markedly increased in Adp14-infected cells from days 3 to 4, indicating that these cells had become apoptotic. We confirmed the presence of apoptotic cells in mock-, AdCtrl-, and Adp14-infected H513 cell cultures with the Annexin V apoptosis assay. At 96 hours after infection with Adp14, the number of early apoptotic cells was increased considerably (Annexin V-PE binding and propidium iodide excluding) compared with mock-infected and AdCtrl-infected cells (10.89% versus 4.68% and 4.99%, respectively), which demonstrated that Adp14 induced apoptotic cell death in this cell line.

**Specificity of Adp14ARF Cytotoxicity to Cancer CellsContaining Wild-Type p53**

To confirm that the G1 cell cycle arrest and induction of apoptotic death mediated by Adp14 were dependent on p53 status, we compared the ratios of IC50 values after Adp14 infection relative to the IC50 values of HCT116 wild-type p53 (+/+) and p53-null (−/−) colon cancer cell lines and H28, H513, or H2052 mesothelioma cell lines (8). In both HCT116 cell lines, endogenous expression of p14ARF was not detected by immunoblotting. The four mesothelioma cell lines used in this study had been examined by single-strand conformation polymorphism, and no mutations in exons 5–8 of the p53 gene were detected (data not shown). Although the cytotoxicity of Adp14 varied among individual cell lines, all mesothelioma cells tested were fivefold more sensitive to Adp14 than to AdCtrl. The difference between Adp14 and AdCtrl in their cytotoxicity, measured by the ratio of their IC50 values (IC50 of AdCtrl/IC50 of Adp14), was statistically significantly lower in HCT116 p53-null (−/−) cells than it was in HCT116 wild-type p53 (+/+)((3.0 ± 0.15)-fold versus [10.3 ± 0.99]-fold, respectively; P = .002).

**DISCUSSION**

In human cancers, the frequency that the INK4a/ARF locus is disrupted is second only to the frequency that p53 is disrupted (20). Melanomas, biliary tumors, non-small-cell lung carcinomas, pancreatic carcinomas, and esophageal carcinomas frequently have point mutations in INK4a, whereas mesotheliomas, T- and B-cell acute lymphoblastic leukemias, bladder and nasopharyngeal carcinomas, anaplastic astrocytomas, and glioblastoma multiforme usually have INK4a/ARF deletions (21). Furthermore, the human ARF gene promoter is a CpG island that can be silenced by DNA methylation (22). Several studies (7,8,23,24) have investigated p16INK4a gene replacement therapy for various human cancer cells. However, much less is known about the p14ARF-mediated effects in human cancers. In this study, we demonstrated that adenovirally transferred p14ARF gene resulted in increases of both p53 and p21WAF proteins, dephosphorylation of pRB, subsequent arrest of cells at the G1/S-phase transition, and eventual apoptotic cell death in human mesothelioma cells. Although we did not examine the INK4a/ARF locus in these mesothelioma cell lines, the absence of both p16INK4a and p14ARF expression, as judged by immunoblotting assays, suggested defective expression of these two regulators. Although overexpression of ARF can induce both G1- and G2-phase arrest (5), our data show that Adp14-infected mesothelioma cells are predominantly arrested in G1 phase. In normal cells, pRB regulates cell proliferation by binding and sequestering transcription factors essential for progression to S phase. These transcriptional factors are released in late G1 phase by the cyclin D-dependent kinase-mediated phosphorylation of pRB, thereby allowing cells to enter S phase (1,19). Unlike p16INK4a.
which inhibits only the cyclin D-dependent kinases, p53-induced p21WAF can induce G1 arrest by inhibiting the cyclin D-, E-, and A-dependent kinases (19). Furthermore, MDM2 and pRB can also interact physically through direct complex formation, which can relieve pRB-mediated suppression of transcriptional activity (25). Therefore, one consequence of p14ARF overexpression is the induction of G1 arrest by blocking MDM2 inhibition of pRB. Because no mutations or rearrangements of the RB gene have been noted in mesothelioma (26,27), we did not test cells with normal p53 but abnormal pRB.

In addition to the genetic disruption in INK4a/ARF locus, a recent report (28) has demonstrated the presence and expression of simian virus 40 in many mesotheliomas. Simian virus 40 large T antigen can inactivate both pRb and p53 and is considered to act as a carcinogen in mesothelioma (29,30). The interaction between p14ARF and simian virus 40 large T antigen in mesothelioma cells requires further study.

It is clear that p53-mediated apoptosis is modulated by activated oncogene products not found in normal cells. Agents that activate p53 in cancer cells have the potential to kill these cells selectively. These agents would dissociate p53–MDM2 complexes or reactivate inactive forms of wild-type p53, or a wild-type p53 gene could be inserted into a cancer cell (31). The finding that endogenous wild-type p53 expression is a critical determinant of p14ARF-mediated cytotoxicity conflicts with the previous reports that cancer cells with wild-type p53 were resistant to adenoviral vectors expressing p53 (32) and p21WAF (33). The reasons for this discrepancy need to be elucidated; however, the results presented herein suggest that Adp14ARF may be useful for gene therapy for cancers that contain wild-type p53. In addition, tumors lacking functional p53 are, in many cases, refractory to chemotherapy or radiation therapy (34). Adenoviral transfer of p53 gene has been shown to enhance the sensitivity of tumor cells to ionized irradiation (35) and genotoxic agents (36). Because p14ARF overexpression can restore and further increase p53 activity, it may be desirable to combine p14ARF gene therapy with conventional radiation therapy and/or chemotherapeutic agent(s) for the treatment of mesothelioma. Possible synergetic effects for mesothelioma between Adp14 and chemotherapeutic agents are being investigated in our laboratory. Thus, our results suggest that this gene therapy-based approach may be of use in the treatment of mesothelioma.

REFERENCES

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NOTE

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