In Vitro and *in Vivo* Adenovirus-mediated *p53* and *p16* Tumor Suppressor Therapy in Ovarian Cancer[1]

See *The Biology Behind:* M. E. Murphy, The Battle between Tumor Suppressors: Is Gene Therapy Using $p16^{INK4a}$ More Efficacious Than p53 for Treatment of Ovarian Carcinoma? Clin. Cancer Res., *7:* 1487–1489, 2001.

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ABSTRACT

Purpose: **The objectives of this study were to determine the effects of adenovirus-mediated *p16* and *p53* on growth and apoptosis in ovarian cancer cells and on survival in nude mice implanted with human ovarian cancer cells.**

Experimental Design: **SKOV-3 ip1 (*p53* and *p16* null), 2774 (*p53* and *p16* mutant), and OVCA 420 (*p53* and *p16* wild-type) cells were used for *in vitro* studies. SKOV-3 ip1, 2774, and Hey A8 (*p53* and *p16* wild-type) cells were used in the nude mouse studies. The E1-deleted adenoviruses containing *p53*, *p16*, or β-*galactosidase* cDNA were transfected into the different cell types or inoculated into the nude mice after injection with ovarian cancer cells.**

Results: **Cell counting, microtetrazolium, and anchorage-independent growth assays on transfected cells demonstrated that *p16* and the *p16*/*p53* combination suppressed growth, whereas *p53* did not (except in the anchorage-independent growth assay). Although cells infected with the *p16*/*p53* combination had decreased growth compared with cells infected with either tumor suppressor alone, the difference was only statistically significant compared with *p53*. *p16*, *p53*, and the *p16*/*p53* combination all increased apoptosis in the cells. In the nude mice, *p16* treatment resulted in the longest survival for all three models, although it only reached statistical significance for the 2774 and SKOV-3 ip1 groups.**

Conclusions: **Overall, *p16* demonstrated greater growth inhibition than *p53* both *in vivo* and *in vitro*. The *p16*/*p53* combination demonstrated a consistent trend toward increased growth suppression and apoptosis over *p16* or *p53* alone. Adenovirus-mediated *p16* may be a viable future treatment for ovarian cancer.**

INTRODUCTION

Ovarian cancer is the second most common gynecological malignancy in the United States and is the fifth leading cause of cancer death among women. The disease is usually diagnosed at an advanced stage, and despite aggressive surgical debulking and chemotherapy regimens, the 5-year survival remains a dismal 11–25% for advanced stages (1). Other forms of treatment are clearly needed, and several recent studies have focused on gene therapy as a viable option. Specifically, the adenovirus-mediated introduction of tumor suppressor genes such as *p53* and *p16* is effective in slowing ovarian cancer growth *in vitro* (2, 3).

The tumor suppressor genes *p16* and *p53* have been shown to regulate the cell cycle through different mechanisms. *p53* regulates the cell cycle at the G_1 checkpoint and is primarily stimulated by DNA damage. Activation of *p53* leads to either G_1 arrest or apoptosis; the protein product of *p53* binds to damaged DNA and serves as a transcriptional activator. Specifically, *p53* causes growth arrest through the induction of *p21* and causes apoptosis via activation of *Bax*. p53 is also regulated by *MDM-2*; an increase in *MDM-2* results in inhibition of *p53* activity. In ovarian cancer, *p53* mutations have been found in up to 50% of women with late-stage disease (4, 5). *In vitro* assays have demonstrated that the introduction of the wild-type *p53* gene via a recombinant adenovirus can inhibit growth of ovarian cancer cells, regardless of endogenous *p53* status (3, 6, 7). Several groups have also demonstrated the efficacy of adenovirus-mediated *p53* in slowing ovarian cancer growth *in vivo* (6–9). Ongoing trials are evaluating this treatment in women with recurrent ovarian cancer.

p16 encodes a protein that inhibits cyclin-dependent kinase 4 and cyclin-dependent kinase 6 cyclin D kinases. *p16* is a member of the INK4 cell cycle proteins, and these kinases are required for phosphorylation of the retinoblastoma gene product (RB). Hypophosphorylated *RB* functions to inhibit entry of cells into the S-phase. *In vitro* studies have also demonstrated the efficacy of *p16* transfection via an adenovirus vector in the inhibition of growth and induction of apoptosis in ovarian cancer cell lines (2, 10).

The objective of this study was 2-fold. The first objective was to determine whether the transfection of *p16* and *p53* together resulted in greater growth inhibition and apoptosis in ovarian cancer cell lines than either individually. The second

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purpose was to evaluate the effect of that transfection on survival in a nude mouse ovarian cancer model.

MATERIALS AND METHODS

Cell Lines. SKOV3ip1 (*p16* and *p53* null), 2774 (*p16* and *p53* mutant), and OVCA 420 (*p16* and *p53* wild-type) cells were grown in MEM supplemented with 10% fetal bovine serum. The OVCA 420 cell line does not grow *in vivo*, and Hey A8 cells (*p53* and *p16* wild-type), along with SKOV-3 ip1 and 2774, were used for the nude mouse model studies.

Adenovirus Infection. Adenoviral vectors contained wild-type *p53*, *p16*, or *Escherichia coli* β-*gal*[3] cDNA and a CMV promoter inserted into the E1-deleted region of modified adenovirus (Ad5CMV). Monolayer cells were grown in MEM with 10% fetal bovine serum and were infected with adenoviral vectors at a multiplicity of infection of 100 for all subsequent experiments. Cells receiving both *p16* and *p53* were infected with both vectors at an multiplicity of infection of 100.

Growth Suppression Assays. Cells were initially plated in triplicate in MEM at a density of 2×10^4 cells/well. Cells were either uninfected (mock); infected with Ad5CMVβ-gal, Ad5CMVp53, AD5CMVp16; or both Ad5CMVp53 and Ad5CMVp16. Cells were harvested and counted with a Coulter counter on days 1, 3, 4, 5, 6, and 7 after infection (Coulter Corp., Miami, FL). Each growth assay was completed in triplicate for three different experiments. The average number of cells/ml for each day after infection was calculated by combining data from all nine experiments. Growth was also monitored using dye conversion in a MTT assay as described previously (11).

Anchorage-independent Colony Formation. Cells from the three cell lines were infected 24 h after plating. Twenty-four h after infection, 1×10^3 cells were plated in triplicate in 0.3% agar in three separate experiments. Plates were incubated at 37°C, and colonies were counted on an inverted microscope at ×4 magnification at 3 weeks after plating. Total numbers of colonies/dish and colony-forming efficiency were calculated.

Protein Expression. Total cell lysates were prepared by sonicating cells 3 days after infection in RIPA buffer (150 mM NaCl, NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0). Fifty μg of protein were loaded onto SDS-polyacrylamide gels (gels ranged from 10–13%, depending on protein to be evaluated) and electrophoresed. The protein was then transferred to a Hybond-ECL membrane (Amersham, Arlington Heights, IL). Membranes were blocked with 5% nonfat dry milk and 0.1% Tween 20 and probed with antibodies against the proteins of interest. The specific antibodies used were mouse antihuman *p53* (Amersham), mouse antihuman *p16* (Oncogene Research Production, Cambridge, MA), rabbit anti-*Bax* (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-*Bcl-2* (DAKO, Carpinteria, CA), and mouse anti-*RB* (Santa Cruz Biotechnology). Appropriate IgG horseradish peroxidase-conjugated goat antimouse or antirabbit secondary antibodies were used, and blots were processed per the manufacturers' suggestions.

Apoptosis. Evidence for apoptosis was evaluated via a terminal deoxynucleotidyltransferase-mediated nick end labeling-based assay (Apo-BRDU kit; Phoenix Flow Systems, San Diego, CA). Plates of 1×10^6 cells were either then mock infected or infected with Ad5CMVβ-gal, Ad5CMVp53, AD5CMVp16, or both Ad5CMVp53 and Ad5CMVp16 at the same multiplicity of infection as the growth curves and evaluated 3 days later. For evaluation, cells were initially fixed in 1% formaldehyde and then in 70% ethanol. They were then incubated with terminal deoxynucleotidyltransferase enzyme and Br-dUTP, rinsed, and incubated with fluorescent monoclonal antibody and PI staining buffer (50 mg/ml PI and 15 mg RNase). The percentages of positive cells were determined by flow cytometry (Coulter Epics XL-MCL; Coulter Corp.).

Cell Cycle Analysis. Cells were incubated with adenoviral vectors for 72 h at a density of 1×10^6 cell/10-ml plate. Cells were then fixed in ethanol and stored at 4°C. At the time of cell cycle analysis, cells were washed and resuspended in PI staining buffer. DNA content was evaluated by flow cytometry (Coulter Epics XL-MCL).

Nude Mouse Model. This protocol was approved by the Animal Care and Utilization Committee at The University of Texas M. D. Anderson Cancer Center. Seventy-five female, athymic nude mice (Harlan Sprague Dawley Inc., Indianapolis, IN) were obtained at 6–8 weeks of age and kept in a contained biohazard area. The mice were divided into three groups of 25 mice. Each group was injected i.p. with either Hey A8, SKOV-3 ip1, or 2774 cell lines (5×10^6cells in 200 μl of sterile saline). Within each group, there were five different treatment groups of five mice each. Each group of five mice received i.p. injections of either saline, β-*gal* virus, *p16* virus, *p53* virus, or both *p16* and *p53* adenoviruses. Viral doses (2×10^9 plaque-forming units/200 μl of saline per injection) were given on days 4, 6, and 8 after inoculation with the ovarian cancer cells. Mice were then evaluated daily for morbidity and mortality. Mice were euthanized once the ascites was large enough to preclude movement or if cachexia produced severe morbidity. Ascitic fluid, tumor samples, and liver samples were collected at the time of necropsy. All surviving mice were sacrificed 150 days after initial ovarian cancer cell inoculation. Multiple biopsies of tissue from mice lacking gross evidence of disease at necropsy were evaluated for evidence of cancer by a veterinary histopathologist.

Statistical Analysis. SPSS 10.0 software (SPSS, Inc., Chicago, IL) was used for statistical analysis. One-way ANOVA was used to compare means between treatment groups and Tukey's HSD was used to evaluate the statistically significant differences between groups. The Kaplan-Meier method and the log-rank statistic were used to evaluate survival in the nude mouse model. $P < 0.05$ was deemed significant.

RESULTS

Adenovirus Infection. Western blot analysis detected *p16* and *p53* protein products in all cell lines after infections with adenoviral constructs; this was done to confirm expression of infected proteins as well as to establish baseline protein expression patterns. *p53* was not expressed in the SKOV-3 ip1

[3] The abbreviations used are: β-gal, β-galactosidase; CMV, cytomegalovirus; MTT, microtetrazolium; PI, propidium iodide; HSD, honestly significant difference; NED, no evidence of disease.

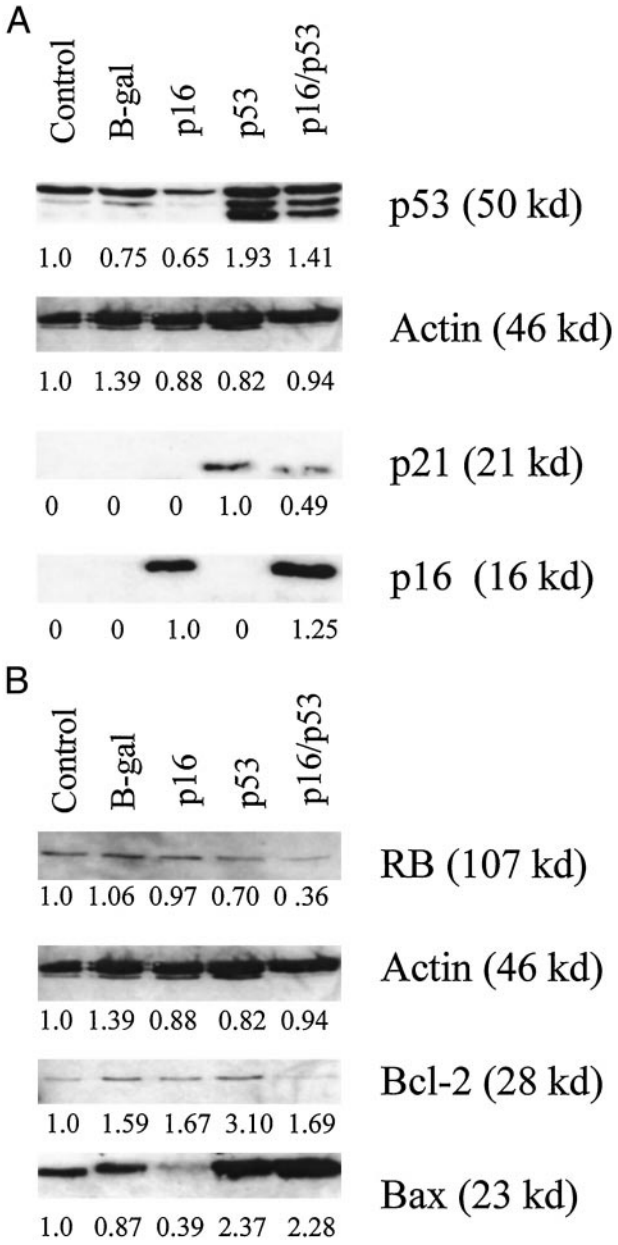

Fig. 1 *A*, expression of *p16, p21, p53,* and *actin* controls after infection of 2774 cells with controls and the appropriate adenovirus. Western blot analysis was performed with 50 μg of protein and probing with the antibody of interest. Relative densitometry data compared with controls are listed for each lane. *B*, expression of *RB, bcl-2, bax,* and *actin* controls after infection of 2774 cells with controls and the appropriate adenovirus. Western blot analysis was performed with 50 μg of protein and probing with the antibody of interest. Relative densitometry data compared with controls are listed for each lane.

line (*p53* null) and expressed at low levels in the 2774 and 420 (p53 mutant and wild-type, respectively) cell lines. *p53* was always expressed strongly when it was transfected; there was not a difference in levels between the *p53*- and the *p16*/*p53*-transfected groups. p16 was only expressed in cell lines that were infected with the *p16*-containing adenovirus; it was expressed less in the *p16* group than in the p16/p53 group (Fig. 1).

Growth Suppression Assays. All data were pooled and analyzed to determine the overall effect of the five treatment types (mock, β-*gal*, *p16*, *p53*, and *p16*/*p53*) on growth, as assessed by one-way ANOVA comparing the average cell counts on designated days after infection. There was no significant difference on day 1 ($F = 0.297$; $P = 0.879$), however, on days 3, 4, 5, 6, and 7, there was a statistically significant difference between the groups ($F = 3.5$–26; $P = 0.000$–0.011). Tukey's HSD test showed that by day 3, only the *p16*/*p53* group showed decreased growth as compared with mock and β-*gal* ($P = 0.030$–0.034). By day 7, both the *p16* and *p16*/*p53* had decreased growth compared with the mock, β-*gal*, and *p53* groups ($P = 0.000$–0.047). The *p53* did not demonstrate a statistically significant decrease in cell growth as compared with controls. Although the *p16*/*p53* group did have a lower mean number of cells/ml than the *p16* group, it was not statistically significant ($P = 0.117$–0.995; Fig. 2).

The data were also analyzed separately for each cell type, and only minimal exceptions to the results of the pooled data were found. In SKOV-3 ip1 cells; the only difference was that growth was more suppressed in the *p53* and β-*gal* groups than the mock group by day 7 ($P = 0.004$ and 0.008, respectively). In OVCA 420 cells, both *p16* and *p16*/*p53* groups showed decreased growth compared with the mock and β-*gal* groups on days 4 and 6 ($P = 0.000$–0.016), but by day 7, the differences had lost statistical significance ($F = 1.709$; $P = 0.167$). In 2774 cells, *p16* and *p16*/*p53* groups showed significantly less growth than mock and β-*gal* groups at days 3 and 6 ($P = 0.003$–0.039); however, that difference also disappeared by day 7.

The results from the MTT assay were remarkably similar to those in the cell counting assays. Using the pooled data, for all 4 days (days 1, 3, 6, and 7) significant differences were found among treatment groups ($F = 5.3$–39.8; $P = 0.000$–0.001). The *p16* and the *p16*/*p53* groups showed significantly decreased cell growth than mock and β-*gal* on all days tested ($P = 0.000$–0.031). The *p53* group did not differ from controls, although it was always significantly different from both the *p16* and *p16*/*p53* groups. Although the *p16*/*p53* group always had a lower mean uptake than the *p16* group, it was only statistically significant on day 3 ($P = 0.011$; Fig. 3).

The MTT data for the individual cell lines were almost identical to the pooled data, except that the data on day 1 were not significantly different among the groups. In the SKOV-3 ip1 cell line, the *p16* and *p16*/*p53* groups differed by day 7 ($P = 0.001$). In the 420 cell line, growth in the *p53* group was decreased compared with that of controls by day 7 ($P = 0.005$–0.020).

Anchorage-independent Colony Formation. One-way ANOVA revealed a significant difference between the mean colony-forming efficiencies of the various treatment groups ($F = 100$; $P = 0.000$). All three intervention groups resulted in decreased colony-forming efficiency compared with both the mock-infected and the β-*gal*-infected groups ($P = 0.000$ for p16, p53, and p16/p53). Cells containing the β-*gal* construct did have a lower mean colony-forming efficiency than the mock control, but it was not statistically significant ($P = 0.206$). The *p53* group had more growth inhibition than the controls; however, the *p16* and the *p16*/*p53* groups had even greater growth inhibition than the *p53* group ($P = 0.000$ for both). Although the

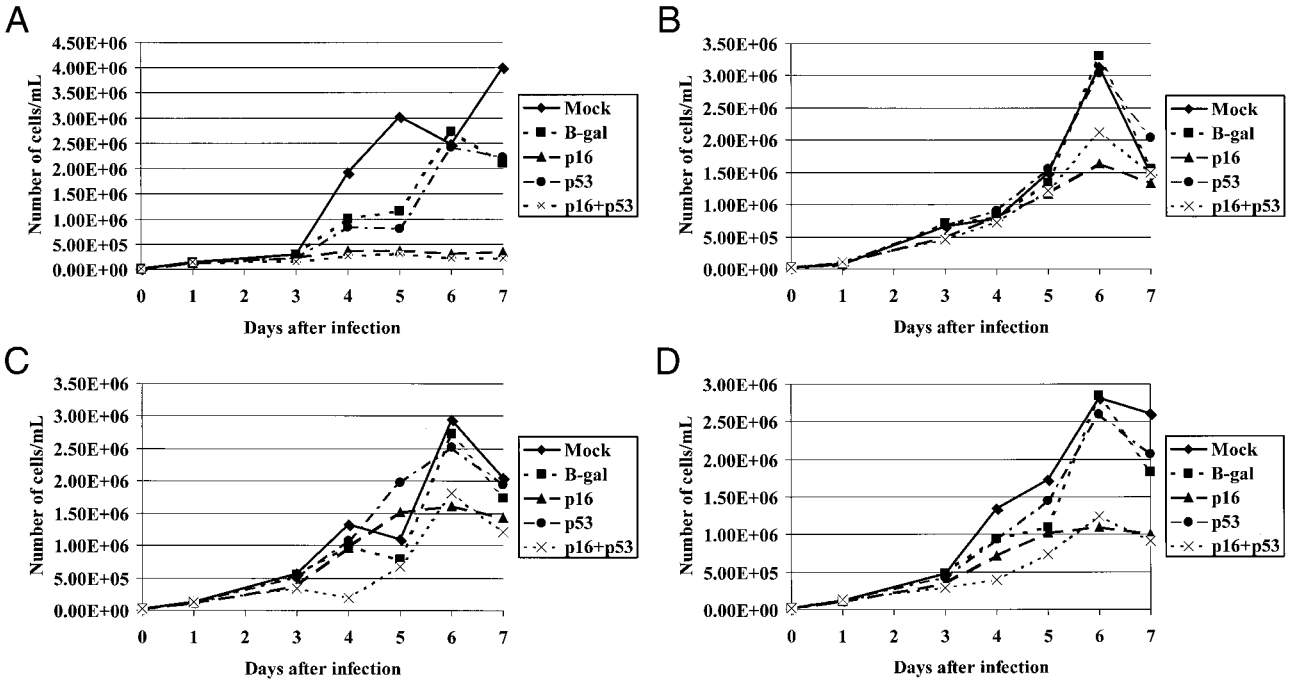

Fig. 2 *A*, SKOV-3 ip1 cell counting assay that demonstrates decreased growth after infection with adenovirus containing *p16* or both *p16* and *p53*. Curves represent average growth curves after infection with adenovirus vectors for nine total experiments. *B*, 2774 cell counting assay that demonstrates decreased growth after infection with adenovirus containing *p16* or both *p16* and *p53*. Curves represent average growth curves after infection with adenovirus vectors for nine total experiments. *C*, 420 cell counting assay that demonstrates decreased growth after infection with adenovirus containing *p16* or both *p16* and *p53*. Curves represent average growth curves after infection with adenovirus vectors for nine total experiments. *D*, cell counting assay that demonstrates decreased growth after infection with adenovirus containing *p16* or both *p16* and *p53*. Curves represent average growth curves for all three ovarian cancer cell lines after infection with adenovirus vectors for nine total experiments.

p16/*p53* group had a fewer colonies than the *p16* group, the difference was not statistically significant (Table 1).

Protein Expression. The *retinoblastoma* gene product was expressed in all three cell lines and did not appear affected by the transfection with p16 or p53. *Bax* expression was found in both controls and infected cells; however, it was enhanced after transfection with *p53* and suppressed after transfection with *p16*. *Bcl-2* expression was demonstrated only in the 2774 cell line and was unaffected by transfection with the various adenoviral constructs. *p19*, another member of the INK4 protein family, expression was not detected in any of the cell lines tested. *p21* expression was induced in the cell lines after infection with *p53* (Fig. 1).

Apoptosis. When data were pooled, one-way ANOVA showed that the *p53*, *p16*, and *p16*/*p53* groups had a significantly increased percentage of cells in apoptosis than mock and β-*gal* controls ($F = 31.1$; $P = 0.000$). The *p16*/*p53* group had significantly more apoptosis than the *p16* group ($P = 0.000$) but not more than the *p53* group, although it showed a trend toward significance ($P = 0.107$). These trends were consistent with those when each cell line was analyzed individually; the only exception was in the SKOV-3 ip1 line in which only the *p53* group demonstrated a significant increase in apoptosis ($P = 0.020$; Table 2).

Cell Cycle Analysis. The pooled data from all three cell lines revealed a significant difference in the percentage of cells in G_1 ($F = 4.32$; $P = 0.002$). Further analysis revealed that the major difference was a lower percentage of cells in G_1 in the β-*gal* group (44%) than the mock (56%), *p53* (54%) or *p16* (57%) groups ($P = 0.005–0.038$). The percentage of cells in G_1 also differed between the *p16*/*p53* and the β-*gal* groups but was not significant (53% *versus* 44%; $P = 0.067$). When the three cell lines were analyzed individually, the β-*gal* group consistently had a lower percentage of cells in G_1 than did the other groups; otherwise, the individual data were inconsistent with the pooled data. For example, in the SKOV-3 ip1 cells, the *p16* and the *p53* groups had a higher G_1 percentage than mock ($P = 0.038$ and 0.001, respectively); the p16/p53 did also but it did not reach statistical significance ($P = 0.066$). In 2774 cells, the *p53* and *p16*/*p53* instead had lower G_1 percentages than mock (28% and 41% *versus* 53%; $P = 0.000$ and 0.029, respectively). Finally, in the 420 cells, only the β-*gal* significantly decreased the percentage of G_1 cells in comparison with the other groups (Table 3).

Nude Mouse Model. For all three cell lines, the *p16* group had the longest survival and the most mice that showed NED at the completion of the experiment. (Table 4; Fig. 4). No control mice in any of the cell lines were found to be histologically NED at the completion of the experiment. No statistically significant differences were found between treatment groups with regard to weight or amount of ascites at the time of necropsy.

In the mice injected with the Hey A8 cell line, all tumor suppressor treatment groups had longer survival than either

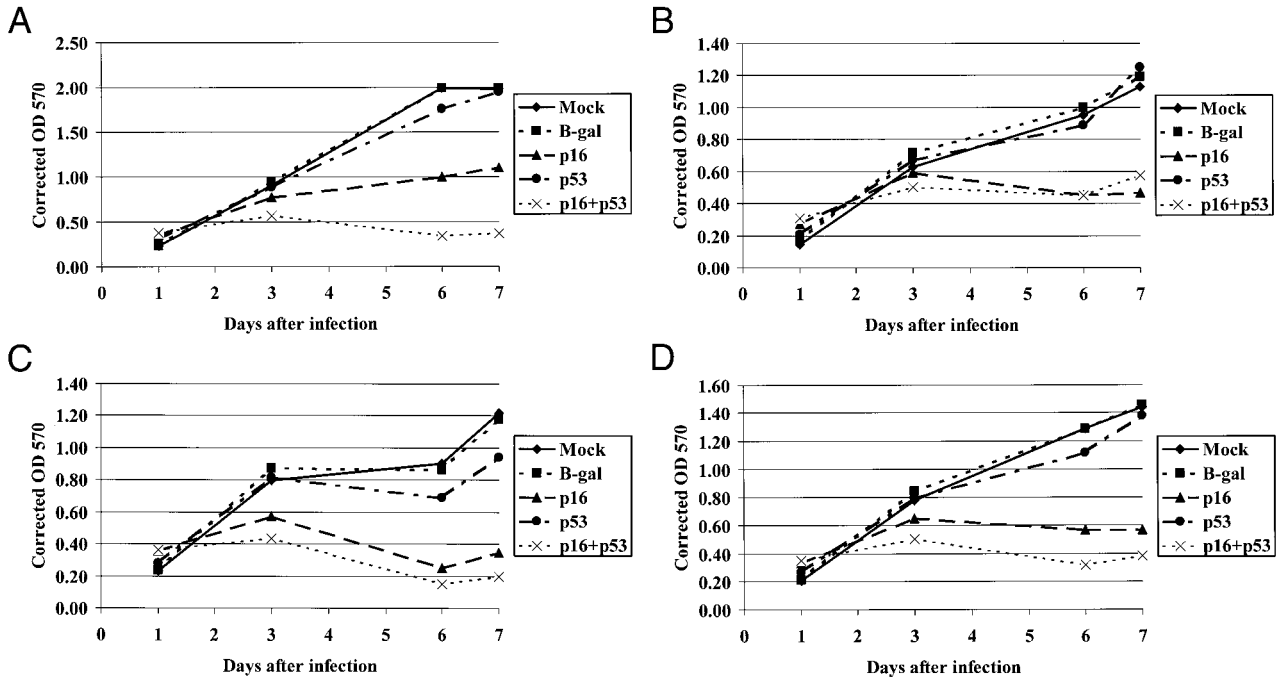

Fig. 3 *A*, SKOV-3 ip1 MTT assay that also demonstrates decreased growth after infection with adenovirus containing *p16* or both *p16* and *p53*. Curves represent average MTT after infection with adenovirus vectors for nine total experiments. *B*, 2774 MTT assay that also demonstrates decreased growth after infection with adenovirus containing *p16* or both *p16* and *p53*. Curves represent average MTT after infection with adenovirus vectors for nine total experiments. *C*, 420 MTT assay that also demonstrates decreased growth after infection with adenovirus containing *p16* or both *p16* and *p53*. Curves represent average MTT after infection with adenovirus vectors for nine total experiments. *D*, pooled data MTT assay that also demonstrates decreased growth after infection with adenovirus containing *p16* or both *p16* and *p53*. Curves represent average MTT for all three ovarian cancer cell lines after infection with adenovirus vectors for nine total experiments.

Table 1 Efficiency of colony formation in soft agarose

	% (*P*)			
Adenovirus	SKOV-3 ip1	OVCA 420	2774	Pooled
Mock infected	99	51	69	73
β-*gal*[a]	88 (0.38)	42 (0.078)	58 (0.241)	63 (0.206)
p53[b]	54 (0.000)	12 (0.000)	18 (0.000)	28 (0.000)
p16[b]	0.5 (0.000)	0.2 (0.000)	1 (0.000)	0.6 (0.000)
p16 and *p53*[b]	0.27 (0.000)	0.05 (0.000)	0.05 (0.000)	0.1 (0.000)

[a] *P* compared with mock control.

[b] *P* compared with mock and β-*gal* controls. *p16* and *p16/p53* were significantly different from *p53* in all cell lines ($P = 0.000-0.028$). *p16* did not differ significantly from *p16/p53* ($P = 1.0$).

β-*gal* or mock controls. The differences, however, did not attain statistical significance using the Kaplan-Meier log-rank statistic (2.09; $P = 0.791$) or using ANOVA for length of survival ($F = 0.555$; $P = 0.698$). One *p16*-treated mouse survived 150 days and at the time of necropsy did not have any gross evidence of tumor, and histopathology of numerous biopsies confirmed the absence of microscopic disease.

In the SKOV-3 ip1 cell line, the *p16* group again demonstrated the longest survival; however, there was not a statistically significant difference among the groups using the Kaplan-Meier method and the log-rank statistic (4.40; $P = 0.3544$). However, ANOVA analysis found a significant difference in average survival length between the treatment groups ($F = 2.989$; $P = 0.044$). Further analysis with Tukey's HSD revealed that the difference stemmed from the longer survival of the *p16* group compared with the *p53* group (143 and 85 days, respectively; $P = 0.033$). At the completion of the experiment, 7 mice (1 control mouse, 2 β-*gal* mice, 1 *p53* mouse, 2 *p16* mice, and 2 *p16*/*p53* mice) were grossly free of tumor at necropsy. On final histopathological review of multiple biopsies, the surviving control mouse was found to have adenocarcinoma, whereas all of the surviving treatment mice were still disease free.

In the 2774 cell line, the Kaplan-Meier survival curves were different between the groups (log-rank statistic, 9.56; $P = 0.0485$). Specifically, the β-*gal* group and *p16* groups had a higher survival than mock controls ($P = 0.0018$ and 0.0277, respectively). ANOVA did not show a significant difference in survival length between groups, although it demonstrated a trend ($F = 2.318$, $P = 0.092$). An independent samples *t* test comparing average survival length between mock and *p16* revealed a significant difference (43 and 127 days; $P = 0.007$). A total of 8 mice survived until the completion of the experiment (1 β-*gal* mouse, 4 *p16* mice, 1 *p53* mouse, and 2 *p16*/*p53* mice), and all were free of disease both at necropsy and on histopathology.

DISCUSSION

In light of the poor prognosis for ovarian cancer, the search continues for innovative and efficacious treatment modalities. Along with surgical and chemotherapeutic regimens, gene ther-

Table 2 Mean percentage of cells undergoing apoptosis

	% (*P*)			
Adenovirus	SKOV-3 ip1	OVCA 420	2774	Pooled
Mock	2.4	4.6	1.7	2.9
β-*gal*[a]	1.5 (0.978)	5.1 (1.000)	2.9 (0.997)	3.2 (1.000)
p53[b]	7.0 (0.003–0.020)	34.9 (0.000)	22.9 (0.000)	21.9 (0.000)
p16[b]	1.9 (0.997–0.999)	28.4 (0.000)	21.3 (0.000)	17.2 (0.000)
p16 and *p53*[b]	3.2 (0.785–0.983)	49.0 (0.000)	34.6 (0.000)	28.9 (0.000)

[a] *P* compared with mock control.

[b] *P* compared with mock and β-*gal* controls. *p16* and *p53* values were not significantly different except in the SKOV-3 ip1 ($P = 0.004$). *p16* and *p16/p53* were significantly different ($P = 0.000–0.008$) except in SKOV-3 ip1 ($P = 0.889$). *p53* and *p16/p53* were significantly different in OVCA 420 and 2774 ($P = 0.008–0.011$) but not in SKOV-3 ip1 or pooled data ($P = 0.06–0.107$).

Table 3 Cell cycle analysis: percentage of cells in G_1

	% (*P*)			
Adenovirus	SKOV-3 ip1	OVCA 420	2774	Pooled
Mock	63	53	53	56
β-*gal*[a]	55 (0.228)	40 (0.034)	39 (0.005)	44 (0.005)
p53[a]	77 (0.001)	54 (0.999)	28 (0.000)	54 (0.983)
p16[a]	73 (0.038)	50 (0.977)	46 (0.433)	53 (0.999)
p16 and *p53*[a]	72 (0.066)	44 (0.256)	41 (0.029)	49 (0.935)

[a] *P* compared with mock control.

apy has emerged as one of the leading contenders for a place in the treatment armamentarium. Several groups have reported both *in vitro* and *in vivo* success in introducing tumor suppressor genes to slow the growth of ovarian cancer and induce apoptosis (2, 3, 6–10).

The majority of gene therapy research in ovarian cancer has focused on *p53*. *p53* is abnormally expressed in approximately 50–67% of ovarian cancer patients and also appears to be the most frequent genetic alteration in the disease (4, 5). Earlier work by our laboratory and others has demonstrated that *p53* introduced via an adenovirus vector can suppress growth and induce apoptosis in ovarian cancer cell lines (3, 7). The data in this study showed that adenovirus-mediated *p53* increased apoptosis and decreased anchorage-independent growth in the three ovarian cancer cell lines tested. In contrast to some of the previous data in our lab, *p53* was not effective in slowing ovarian cancer growth in the cell counting or MTT assays. This seeming contradiction may be partially explained by the use of a lower multiplicity of infection in the SKOV-3 ip1 line compared with previous work (100 *versus* 250), and the other possibility may stem from potential inconsistencies in viral titers from different viral batches. Unfortunately, *p53* did not increase survival in the nude mouse model, either alone or in combination with *p16*; this may be because of the decreased magnitude of growth inhibition by *p53* when compared with *p16* and the *p16/p53* combination. Several studies have shown that both *p53* and β-*gal* introduced via an adenovirus vector result in increased survival over controls in mouse models (6, 9); one study has shown that *p53* increased survival over both β-*gal* and controls (8).

In ovarian cancer, mutations of the *p16* gene are relatively rare, although *p16* mutations may approach 50% in ovarian cancer cell lines (12–16). Recently, alterations in *p16* protein expression have also been demonstrated (17, 18). One group found no *p16* protein expression in 26% of ovarian cancer tumors studied (17). Similarly, Dong *et al.* (18) found that 11% of ovarian cancers did not express *p16* and that increased *p16* expression correlated with progression and unfavorable prognosis. The mechanism for the decreased *p16* expression in these two studies has not been identified; however, it does not appear to be secondary to hypermethylation. Further work needs to be done to delineate the role of *p16* in the origination and progression of ovarian cancer.

Table 4 Survival data in nude mouse model

Adenovirus	SKOV-3 ip1	Hey A8	2774
Mock	104 (0)	38 (0)	43 (0)
β-*gal*	128 (20)	37 (0)	89 (20)
p53	85 (20)	53 (0)[b]	66 (20)
p16	143 (40)[c]	60 (20)	127 (80)
p16/p53	119 (40)	56 (0)	82 (40)

[a] Mean survival in days (% of NED mice at 150 days).

[b] One mouse became paralyzed and was euthanized at 99 days but had NED.

[c] One mouse died of unknown causes at 145 days but had NED.

Our data support the premise that *p16* is more effective than *p53* or controls in suppressing ovarian cancer cell growth. Other investigators have also demonstrated increased cell cycle arrest, growth inhibition, and apoptosis after adenovirus-mediated transduction of *p16* in gliomas and lung, pancreas, liver, and head and neck tumor cell lines (10, 19–24). Schreiber *et al.* (10) have compared the efficacy of various cyclin kinase inhibitors (*p16, p18, p19, p21,* and *p27*) and found that although they all inhibit growth and increase apoptosis *in vitro*, only *p16* slows tumor progression *in vivo*. Surprisingly, *p16* is also effective in the induction of apoptosis, although the mechanism remains unclear and may be attributable to actions of *p16* that are outside the cell cycle regulatory pathway. Other investigators have also proposed that *p16* may have other functions aside from cell cycle regulation; for example, *p16* was shown to reduce the expression of vascular endothelial growth factor and angiogenesis *in vivo* while having no effect on cell growth in gliomas (20).

Here we report the first *in vivo* experiment to evaluate the effect of treating an ovarian cancer nude mouse model with *p16* via an adenovirus vector. The results demonstrated that *p16* was the only treatment effective in prolonging survival. Similar findings in head and neck cancers and other cell lines show that *p16* can significantly decrease the size of established tumors in nude mice (10, 21). Adenovirus-mediated *p16* appears to be more effective than *p53* or adenovirus alone, although the adenovirus alone did demonstrate some tumoricidal activity. The question of adenoviral vectors having independent action is a chronic issue in gene therapy studies. Unfortunately, it is a question that is difficult to address in a clinical setting because trials with vector alone raise serious ethical considerations. Further work needs to be done in this model to pave the way for future trials in women with recurrent ovarian cancer.

Because *p16* and *p53* act through separate pathways, it may

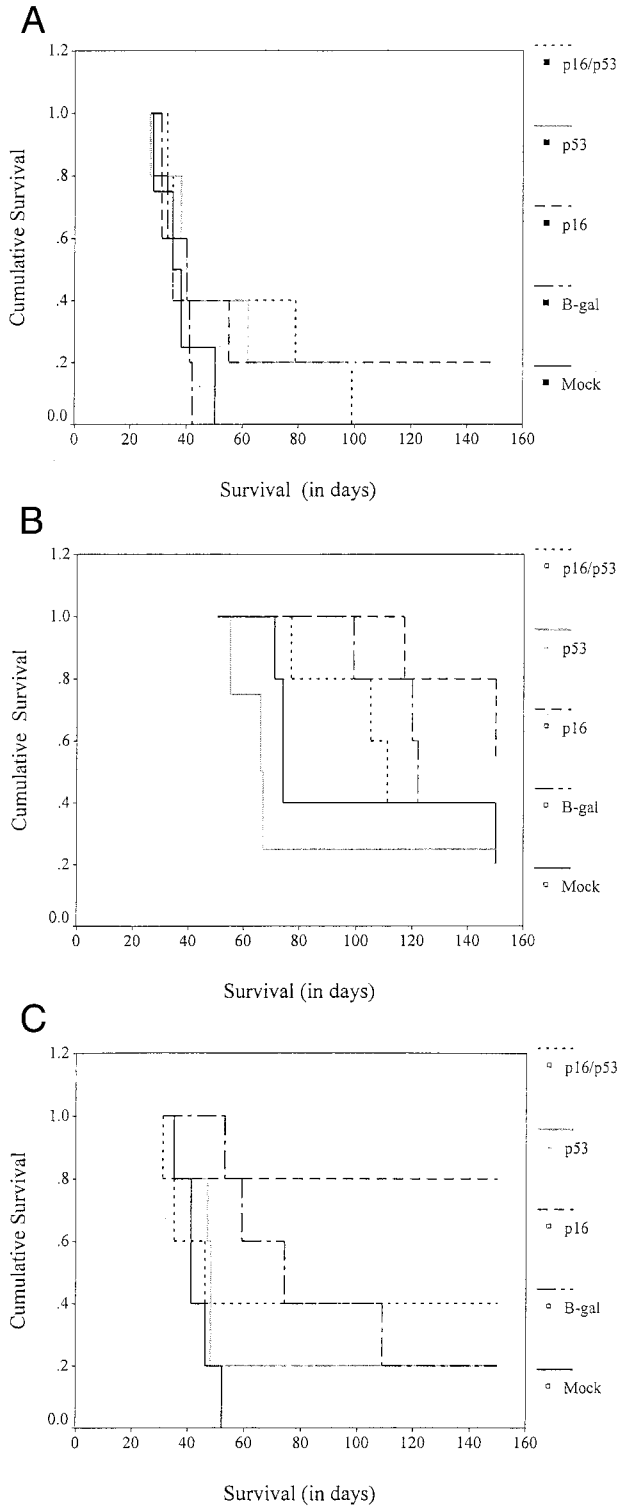

Fig. 4 *A*, Kaplan-Meier survival curve for Hey A8-inoculated nude mice after treatment with adenovirus vectors. *B*, Kaplan-Meier survival curve for SKOV-3 ip1-inoculated nude mice after treatment with adenovirus vectors. *C*, Kaplan-Meier survival curve for 2774 inoculated nude mice after treatment with adenovirus vectors.

be beneficial to combine the two constructs to enhance their therapeutic benefit. Our data showed that the *p16*/*p53* combination increased the percentage of cells undergoing apoptosis and inhibited growth more than either tumor suppressor alone. Additional benefit demonstrated in the *in vitro* assays did not translate into increased survival in the nude mouse model. However, the only other *p16*/*p53* combination study in the literature has reported that the *p16*/*p53* combination can increase apoptosis and that infected cancer cells cannot form tumors in the nude mouse model (25). More work is necessary to further delineate the complex set of cellular events that are initiated by these two tumor suppressors. The present results lend credence to the idea that *p16* may be superior to *p53* in gene therapy for ovarian cancer.

Combination therapy is gaining popularity in attempts to improve survival in ovarian cancer. Heretofore, most of the effort has been geared toward identifying chemotherapeutic agents that act synergistically to improve outcome. Gene therapy may prove to be a useful adjunctive treatment to complement current therapies. Future work might focus on combining *p53* with other agents, perhaps tumor suppressors such as *p16* or chemotherapeutic agents such as cisplatin or paclitaxel. Preliminary work with *in vitro* and *in vivo* adenovirus-mediated *p53* transfection in combination with paclitaxel, cisplatin, doxorubicin, 5-fluorouracil, methotrexate, or etoposide in human head and neck, ovarian, prostate, and breast cancer demonstrated greater anticancer effects than with any of the agents alone (26, 27). In contrast, the work with *p16* in combination with chemotherapy has been far less encouraging. Work with bladder cancer cells and human glioma cells has demonstrated that adenovirus-mediated *p16* transfer actually results in chemoresistance to several agents, including platinum agents, paclitaxel, topotecan, and carmustine (28–30).

In conclusion, the adenovirus-mediated transduction of *p16* is more efficacious than that of *p53* in suppressing growth and increasing survival in the nude mouse model. Although more research is required to characterize the optimal dosages and protocols, adenovirus-mediated *p16* gene therapy may soon be a viable treatment option for ovarian cancer patients.

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