Bcl-2 Antisense Oligonucleotide Overcomes Resistance to E1A Gene Therapy in a Low HER2-Expressing Ovarian Cancer Xenograft Model

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Abstract
We are currently conducting clinical trials of E1A gene therapy for patients with ovarian cancer. The adenovirus type 5 E1A gene suppresses growth of ovarian cancer cells that overexpress HER-2/neu (HER2) and growth of some—but not all—that express low HER2. In HER2-overexpressing cells, suppression by E1A is predominantly by down-regulation of HER2, but the mechanism in low HER2-expressing cells is not fully understood. The adenoviral E1B protein has sequential and functional homology to Bcl-2 and prolongs the viability of adenovirus host cells by inhibiting E1A-induced apoptosis. Bcl-2 is overexpressed in ovarian cancer and participates in chemoresistance; we hypothesized that Bcl-2 inhibits E1A-induced apoptosis leading to resistance to E1A gene therapy. E1A suppressed colony formation of ovarian cancer cells that express low levels of Bcl-2 and HER2 (OVCAR-3 and OVCA 433), but enhanced colony formation in low HER2-, high Bcl-2–expressing ovarian cancer cells (2774 and HEY). Treating 2774 or HEY cells with antisense oligonucleotide Bcl-2 (Bcl-2-ASO) did not reduce cell viability. E1A combined with Bcl-2-ASO led to significant decreases in cell viability resulting from increased apoptosis relative to cells treated with E1A alone (P < 0.05). The increase in apoptosis was partly due to cytochrome c release and subsequently caspase-9 activation by Bcl-2-ASO. Finally, in an ovarian cancer xenograft model, treatment with Bcl-2-ASO did not prolong survival, but E1A plus Bcl-2-ASO did (P < 0.001). In conclusion, ovarian tumors overexpressing Bcl-2 may not respond well to E1A gene therapy, but treatment with a combination of E1A and Bcl-2-ASO may overcome this resistance. (Cancer Res 2005; 65(18): 8406-13)

Introduction
Ovarian cancer is a major cause of death among women worldwide. In the United States, 25,580 new cases and 16,090 deaths are expected in 2004, accounting for 6% of all cancer deaths in women (1). The HER-2/neu (HER2) gene, which encodes a 185 kDa transmembrane growth factor receptor tyrosine kinase (2), is overexpressed in 20% to 25% of human ovarian cancers and is a marker of poor prognosis (3). The adenovirus type 5 gene E1A has been shown to suppress tumorigenicity by inhibiting HER2 overexpression in the rat embryo fibroblast cell line Rat-1 and in human breast cancer cells (4, 5) and in the HER2-overexpressing ovarian cancer cell lines SKOV3ip1 (6) and SKOV3 (7). Although E1A could suppress the growth of low HER2-expressing cells (8), it failed to improve the survival of female nu/nu mice given an i.p. injection of low HER2-expressing 2774 ovarian cancer cells (6). Other mechanisms, such as suppression of angiogenesis (9), inhibition of metastasis (7, 10, 11), down-regulation of the Axl receptor (12), and induction of apoptosis in cancer and noncancerous cells (9, 13, 14), have been shown to contribute to the antitumor activity of E1A. Apoptosis seems to be mediated by p53-dependent and p53-independent induction mechanisms (15). The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), which is known to induce apoptosis in cancer cells, is sensitized by E1A through the enhancement of caspase activation (16).

Apoptosis induced by E1A can be inhibited by the presence of the adenoviral gene E1B, which can prolong the viability of host cells through this mechanism (13, 17). The E1B gene encodes two oncoproteins, each of which can promote transformation in association with E1A (18). The 55 kDa E1B protein associates with p53 (19), and the 19 kDa E1B protein has sequential and functional homology to the antiapoptotic protein Bcl-2 (20). Overexpression of Bcl-2 is associated with chemoresistance in ovarian cancer, perhaps because Bcl-2 protects cells from apoptosis (21). The functional similarity between Bcl-2 and the 19 kDa E1B protein suggests that like E1B, Bcl-2 overexpression in cells may result in resistance to E1A gene therapy. Bcl-2 overexpression may explain, at least in part, the heterogeneity in the response of ovarian tumors to E1A gene therapy. Here, we examined whether high Bcl-2 expression would inhibit the antitumor effect of the E1A gene in low HER2-expressing human ovarian cancer cells and whether down-regulation of Bcl-2 expression by Bcl-2 antisense oligonucleotide (Bcl-2-ASO) would enhance the antitumor effect of E1A in E1A-resistant ovarian cancer cells.

Materials and Methods
Cell lines and culture conditions. The human ovarian cell lines 2774-C10 (denoted hereafter as 2774), OVCA 433, and HEY, and human breast cancer cell lines MDA-MB-435 and MDA-MB-468 were grown in DMEM-Ham’s F-12 medium (DMEM/F12, Life Technologies, Grand Island, NY)
supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin and maintained in a humidified incubator at 37°C containing 5% CO₂. OVCAR-3 cells were grown in RPMI 1640 (Life Technologies) supplemented with 10% FBS and penicillin/streptomycin and maintained in a humidified incubator at 37°C containing 5% CO₂. All six of these cell lines express only basal levels of HER2; the 2774, HEY MDA-MB-435, and MDA-MB-468 express high levels of Bcl-2, and the OVCA 433 cells express low levels of Bcl-2.

**Colony formation assay.** All four human ovarian cancer cell lines (2774, OVCA 433, OVCA 3-433, and HEY) were transfected in 100 mm plates with 10 μg of pAd5E1A-neo or a control vector (psV2-neo; BD Biosciences, Palo Alto, CA), both of which contain a neomycin resistance gene. Briefly, 10 μg of the plasmid was complexed with DC-Chol, a cationic liposome, which was then used to transfect the cells. At 6 hours after transfection, cells were washed in PBS, incubated in fresh medium for 48 hours, and then split in a 1:5 or a 1:10 dilution and plated on 100 mm plates. Cells were selected by culturing in medium containing the neomycin analogue G418 (Sigma, St. Louis, MO) for 3 weeks, after which colonies were stained with 0.5% crystal violet in 20% ethanol and counted.

**Western blot analysis.** Cells were washed with PBS and then lysed in lysis buffer [20 mmol/L Na₂PO₄ (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% aprotinin, 1 mmol/L phenylmethysulfonylfluoride, 100 mmol/L NaF, and 2 mmol/L Na₃VO₄]; Protein concentrations were measured against a standardized control by using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). A total of 20 μg of protein was separated by PAGE on a 12% SDS gel and transferred to an immunoblot polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Non-specific binding on the PVDF filter paper was minimized by using a blocking buffer containing 5% nonfat dry milk and 0.1% v/v Tween 20 in PBS. The treated filter paper was then incubated with the primary antibody (the antiadenovirus type 5 E1A antibody m58 in a 1:500 dilution; PharMingen, San Diego, CA) and the secondary antibody [horseradish peroxidase (HRP)–goat anti-mouse antibody in a 1:5,000 dilution; Jackson Immunoresearch, Westgrove, PA). Other antibodies included anti-Bcl-2 antibody (1:500 dilution; PharMingen), anti–caspase-8 (1:1,000 dilution; Oncogene Research Products, San Diego, CA), anti–cytochrome c (1:1,000 dilution; Cell Signaling, Beverly, MA), and actin (1:5,000 dilution; Sigma–Aldrich Chemical, Co., Saint Louis, MO), and the secondary antibody HRP–goat anti-Syrian hamster (1:2,000 dilution) and the HRP–goat anti-rabbit (1:5,000 dilution) both from Jackson Immunoresearch.

**Annexin staining.** The Annexin V-phycoerythrin (PE) Apoptosis Detection Kit 1 (BD Biosciences PharMingen) was used to assess apoptosis in terms of the externalization of phosphatidylserine residues according to the specifications of the manufacturer. Briefly, trypsinized cells were washed twice with cold PBS and then resuspended in 1× binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, and 2.5 mmol/L CaCl₂] at a concentration of 1 × 10⁶ cells/mL. Then, 100 μL of the solution (1 × 10⁵ cells) was transferred to a 5 mL culture tube, 5 μL of Annexin V-PE was added, and the cells were gently vortexed and incubated in the dark. Finally, 400 μL of 1× binding buffer was added to each tube and the tubes were analyzed with a FACScan cytofluorometer (Becton Dickinson, San Jose, CA) within 1 hour.

**Viral purification.** Recombinant replication-deficient adenoviral vectors were propagated in the human embryonic kidney cell line 293. At 72 hours after infection, cells were harvested and virus was purified with the Adeno-X Virus Purification Kit (BD Biosciences) and stored at −80°C. Virus titer was measured with the Adeno-X Rapid Titer Kit (BD Biosciences). Two types of adenoviral vectors were used: the control vector Ad.E1A(−) (d312), which lacks E1A, E1B, and E3; and Ad.E1A(+), an adenovirus type 5 that contains E1A but lacks E1B and E3.

**Bcl-2 antisense oligonucleotide and Ad.E1A(+) in vitro.** For the in vitro antisense experiments, the ovarian cancer cell lines 2774 (2 × 10⁶ cells/mL) or HEY (1.5 × 10⁶ cells/mL) were plated. The next day, Lipofectin (Invitrogen, Palo Alto, CA) was used to transfect cells in serum-free medium with the oligonucleotide 5’-TCTCCAGGCTGCGCCAT-3’ (Bcl-2-ASO) or with a control oligonucleotide containing a scrambled sequence of the same base composition (SCO; TriLink BioTechnologies, San Diego, CA; final concentration, 150 mmol/L). Five hours later, the serum-free medium was replaced with DMEM/F12 with serum. The next day, cells were exposed to Ad.E1A(−) or Ad.E1A(+) at 10 multiplicity of infection (MOI) in serum-free medium for 1 hour, followed by the addition of DMEM/F12 and a 48-hour incubation, after which cells were harvested for Annexin staining to detect apoptosis, Western blotting for protein expression, and trypan blue exclusion for cell viability. For the Bcl-2 antisense dose-dependence study, cells were transfected with different concentrations of Bcl-2-ASO (0, 50, 100, or 150 mmol/L) and treated with Ad.E1A(+) at 10 MOI the next day. After a 48-hour incubation, cells were stained with trypan blue and the numbers of dead and live cells were counted. For the caspase assays, at the time of adenovirus infection, the caspase-9 inhibitor Z-LEHD-FMK (BD Biosciences, San Jose, CA) was added at 50 μmol/L to the medium. Cells were stained with trypan blue at 24 hours after infection and the numbers of dead and live cells were counted.

**Mitochondrial/cytosolic fractionation.** Mitochondria were isolated from 2774 cells, treated as described above, with the Cytochrome c Releasing Apoptosis Assay Kit (Biovision, Mountain View, CA) according to the protocol of the manufacturer. Both the cytosolic and mitochondrial fractions were analyzed by immunoblotting for cytochrome c.

**Caspase-9 activity assay.** Caspase-9 activity was measured by using a caspase-9 activity kit (R&D Systems, Minneapolis, MN) according to the protocol of the manufacturer. Briefly, 5 × 10⁵ cells were transfected in serum-free medium with Bcl-2-ASO or with the control oligonucleotide SCO (final concentration, 150 mmol/L; TriLink BioTechnologies). Five hours later, the serum-free medium was replaced with DMEM/F12 with serum. The next day, cells were exposed to Ad.E1A(−) or Ad.E1A(+) at 10 MOI in serum-free medium for 1 hour, followed by the addition of DMEM/F12 and a 30-hour incubation, after which cells were harvested, lysed, and placed on ice. The protein concentration of the supernatants was measured after centrifugation. Caspase-9 activity was determined by incubating 100 μg of protein with LEHD-pNA as a substrate at 37°C for 4 hours. Samples were measured at an absorbance of 405 nm.

**Tumorigenicity of 2774 cells and 2774-E1A stable transfectants in nude mice.** Female nu/nu mice were given a single i.p. injection (2 × 10⁶ cells) of parental 2774 cells or the E1A transfectant clones 2774-E3, 2774-E7, 2774-E8, or 2774-NP (neomycin pool; three mice per group). Mice were monitored daily for signs of tumor formation such as ascites, a hunching posture, or loss of s.c. fat and euthanized with an overdose of CO₂ when they appeared moribund. Survival curves were plotted according to the Kaplan-Meier method.

**Ad.E1A(+) therapy in an ovarian cancer xenograft model.** Female nu/nu mice were given a single i.p. injection of 10 × 10⁶ OVCAR-3 cells. Five days later, the mice (five per group) were given i.p. injections of 0.5 mL PBS, 0.5 mL of Ad.E1A(−) solution [titer 10⁸ plaque-forming units (pfu)], or 0.5 mL of Ad.E1A(+) solution (titer 10⁹ pfu) weekly for 16 weeks. Mice were monitored daily and euthanized when they appeared moribund. Survival curves were plotted according to the Kaplan-Meier method.

**Ad.E1A(+) and antisense Bcl-2 therapy in an ovarian cancer xenograft model.** For these experiments, 2774 cells in log-phase growth were trypsinized, washed twice with PBS, and centrifuged at 250 × g. Viable cells were counted and then 2 × 10⁶ viable cells (in 0.5 mL of PBS) were injected into ascetic conditions into the peritoneal cavities of female nude mice. Mice were then randomly assigned to one of five groups (10 mice per group) and treatment was started 4 days later as follows. Group 1 was given i.p. PBS weekly; group 2, i.p. Ad.E1A(+) weekly (500 μL per injection); group 3, i.p. Ad.E1A(−) weekly (500 μL per injection) for 5 weeks; group 4, i.p. Ad.E1A(+) weekly for 5 weeks and SCO on days 4 to 8, 11 to 15, and 18 to 22; and group 5, i.p. Ad.E1A(+) weekly and Bcl-2-ASO on days 4 to 8, 11 to 15, and 18 to 22. The SCO and Bcl-2-ASO were delivered i.p. in 0.5 mL of sterile saline at a dose of 5 mg/kg as previously described (22). When the mice developed signs of tumor development (ascites, hunching, loss of s.c. fat), they were euthanized with an overdose of CO₂ and necropsy was done. Tumors were collected on days 14, 18, 21, and 28, fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA), and embedded in paraffin for immunohistochemical analysis as described below.
Inoculation of nude mice with 10^6 expressing cell lines would translate to antitumor activity. To determine whether this colony suppression effect in the low Bcl-2–forming colonies formed by 2774 (200%) and HEY cells (142%; Fig. 1). To OVCA 433 (27%) cells, but actually increased the number of suppressed colony formation considerably in OVCAR-3 (36%) and number of colonies formed after selection in G418 medium.

Days later began weekly injections of PBS, Ad.E1A (2774 and HEY). The percentage inhibition values were based on the average (OVCAR-3 and OVCA 433), but not in cells that expressed high levels of Bcl-2 (Hey). Here, we found no difference in survival between mice harboring tumors consisting of parental or neomycin-pool control cells (median, 45 days) and those harboring tumors consisting of parental or neomycin-pool control cells (median, 45 days). Mice in the Ad.E1A(+) group showed no evidence of ascites or tumor formation and survived for >12 months (median survival time not reached), verifying that E1A gene therapy suppressed tumor formation in this model (P < 0.05, log-rank test; Fig. 2A).

To rule out the possibility that inefficient transfection by E1A may underlie its inability to have suppressed 2774 cells in a previous study [in which mice bearing tumors consisting of 2774 cells were injected i.p. with Ad.E1A(+); ref. 6], we assessed the tumorigenicity of three variants of 2774 cells that constitutively express E1A (2774-E3, 2774-E7, and 2774-E8), a neomycin vector control (2774-NP), or the parental 2774 cell line. E1A did not extend survival of the mice.

Figure 2. Response to E1A gene therapy differs according to cell type. A, fifteen female nude mice (five per group) were given an i.p. injection of 10^6 OVCAR-3 cells (which express low levels of HER2 and Bcl-2) followed by weekly injections of PBS, Ad.E1A(-), or Ad.E1A(+) for 16 weeks. The Ad.E1A(+) group survived significantly longer than the control and Ad.E1A(-) groups (P < 0.05). B, fifteen female nude mice (three per group) were given an i.p. injection (2 x 10^6 cells) of 2774 ovarian cancer cells (which express low levels of HER2 and high levels of Bcl-2) that had been stably transfected with E1A (clones 2774-E3, 2774-E7, and 2774-E8), a neomycin vector control (2774-NP), or the parental 2774 cell line. E1A did not extend survival of the mice.

Statistical analysis. Data are presented as means ± 1 SD. Means for all data were compared by two-way ANOVA with post hoc testing. Survival distributions were calculated with the Kaplan-Meier method, and the significance of apparent differences in survival distribution between groups was tested with log-rank tests. P < 0.05 was considered statistically significant.

Results

Effects of E1A on colony formation and tumorigenicity of low HER2-expressing ovarian cancer cells. First, to test whether E1A could suppress the tumorigenicity of low HER2-expressing ovarian cancer cells, we transfected four human ovarian cancer cell lines (2774, OVCAR-3, OVCA 433, HEY) and compared the colony formation assay. The OVCAR-3 and OVCA 433 (36%) and OVCA 433 (27%) cells, but actually increased the number of colonies formed by 2774 (200%) and HEY cells (142%; Fig. 1). To determine whether this colony suppression effect in the low Bcl-2–expressing cell lines would translate to antitumor activity in vivo, we inoculated nude mice with 10^6 OVCAR-3 cells i.p. and 5 days later began weekly injections of PBS, Ad.E1A(-), or Ad.E1A(+) for 4 months. All five mice in the PBS control group, and three of the five mice in the Ad.E1A(-) group, developed ascites and died of disseminated abdominal tumors within 9 months (median survival times, 136 days for the PBS group and 262 days for the Ad.E1A(-) group). In contrast, five mice in the Ad.E1A(+) group survived significantly longer than the control and Ad.E1A(-) groups (P < 0.05).

To rule out the possibility that inefficient transfection by E1A may underlie its inability to have suppressed 2774 cells in a previous study [in which mice bearing tumors consisting of 2774 cells were injected i.p. with Ad.E1A(+); ref. 6], we assessed the tumorigenicity of three variants of 2774 cells that constitutively express E1A (2774-E3, 2774-E7, and 2774-E8) and do not show HER2 down-regulation after i.p. injection of 2 x 10^6 cells in nude mice. Here, we found no difference in survival between mice harboring tumors consisting of parental or neomycin-pool control cells (median, 45 days) and those harboring tumors consisting of the E1A transfectants (median, 30 days; P = 0.959; Fig. 2B).

Low Bcl-2 expression correlates with suppression of colony formation by E1A. Hypothesizing that high Bcl-2 expression blocks the antitumor effect of E1A in human ovarian cancer cells, we assessed Bcl-2 expression in four human ovarian cancer cell lines (2774, OVCAR-3, OVCA433, and HEY) and compared the results with those of the colony formation assay. The OVCAR-3 and

Immunohistochemical analysis. Paraffin blocks were sliced in 4 μm sections and deparaffinized. The expression of E1A protein and Bcl-2 in the tumor tissue sections was detected with the LSAB (labeled streptavidin-biotin) Detection kit (DAKO, Carpinteria, CA) as described previously (25). The primary antibodies used were anti-E1A (dilution 1:40; Oncogene Research Products) and anti-Bcl-2 (ready to use; DAKO). Sections were also stained with H&E.

Figure 1. Low Bcl-2 expression level correlates with suppression of colony formation by E1A. The indicated human ovarian cancer cell lines (all four of which express low levels of HER2) were transfected with pAd5E1A-neo (E1A) or the control pSV2-neo vector (neo), after which cells were selected with a medium containing the neomycin analogue G418, stained with crystal violet, and the number of colonies was counted. Transfection with the E1A construct suppressed colony formation by cells that expressed low levels of Bcl-2 (OVCAR-3 and OVCA 433), but not in cells that expressed high levels of Bcl-2 (2774 and HEY). The percentage inhibition values were based on the average of two different experiments. □, neo; ■, E1A.
OVCA433 cells expressed low amounts of Bcl-2, and the 2774 and HEY cells showed high levels of Bcl-2 (Fig. 3A). This expression pattern correlated with the ability of E1A to suppress colony formation (Fig. 1), raising the possibility that high levels of Bcl-2 may participate in the inhibition of the antitumor effect of E1A in human ovarian cancer cells. Therefore, we proceeded to test whether inhibition of the high levels of Bcl-2 would enhance E1A-induced apoptosis.

**Antisense Bcl-2 enhances E1A-induced apoptosis in 2774 ovarian cancer cells.** We next used an antisense oligonucleotide to determine whether inhibition of Bcl-2 would enhance E1A-induced cell death in 2774 and HEY cells. We observed that Bcl-2-ASO suppressed the expression of Bcl-2 within 24 hours and that this suppression was maintained up to 72 hours after treatment (Fig. 3B); a scrambled oligonucleotide (SCO) had no effect on Bcl-2 levels. Cell growth assays of 2774 cells confirmed that infection with Ad.E1A(+) was more cytotoxic than infection with Ad.E1A(-) (P < 0.05) and that the combination of E1A and Bcl-2-ASO significantly enhanced this cytotoxic effect (P < 0.05 versus other treatment conditions; Fig. 4A). Combining Ad.E1A(-) and Bcl-2-ASO suppressed proliferation by only 18% relative to that of Ad.E1A(-) and SCO; however, combining Ad.E1A(+) with Bcl-2-ASO suppressed proliferation by 68% relative to that of Ad.E1A(+) and SCO (P < 0.05). Similar results were obtained with HEY cells (data not shown) and with two human breast cancer cell lines (MDA-MB-435 and MDA-MB-468; data not shown). In other words, the antisense Bcl-2, but not a scrambled oligonucleotide, augmented the cytotoxic effect of E1A in vitro.

To determine whether the enhanced cytotoxicity of E1A in the presence of Bcl-2-ASO in 2774 and HEY cells was attributable to an increase in apoptosis, we used Annexin V-PE staining and found that E1A in combination with Bcl-2-ASO increased the extent of apoptosis by 54% over that produced by E1A plus a scrambled sequence in 2774 cells (Fig. 4B). Bcl-2-ASO plus Ad.E1A(-) had a minimal effect on apoptosis relative to SCO plus Ad.E1A(-). Similar results were obtained in HEY cells (data not shown) and in the breast cancer cell lines MDA-MB-435 and MDA-MB-468 cells (data not shown). These findings suggest that Bcl-2-ASO down-regulated Bcl-2 and enhanced E1A-mediated apoptosis in this high Bcl-2–expressing ovarian cancer cell line. To further confirm this observation, we transfected 2774 cells with various amounts of Bcl-2-ASO (0, 50, 100, and 150 nmol/L) and then infected the cells with a fixed amount of Ad.E1A(+) particles (10 MOI). We found that increasing amounts of Bcl-2-ASO resulted in corresponding decreases in Bcl-2 expression (Fig. 4C). E1A cytotoxicity was also enhanced in a Bcl-2 dose-dependent manner (i.e., the greatest cytotoxicity occurred when Bcl-2 was down-regulated the most—with the highest concentration of Bcl-2-ASO) in combination with E1A (Fig. 4D).

**Apoptotic effect of E1A is enhanced by antisense Bcl-2 through caspase-9 activation via cytochrome c release.** E1A, on its own, has been shown to cleave caspase-8 and to induce the release of cytochrome c from the mitochondria, effects that are inhibited by Bcl-2 in human epithelial cells (24). Hence, we sought to determine whether the combination of Bcl-2-ASO with E1A would enhance the cleavage of caspase-8. Caspase-8 cleavage was not enhanced in the cells treated with E1A and Bcl-2-ASO compared with cells treated with E1A only (Fig. 5A). However, cells treated with a combination of Bcl-2-ASO plus E1A, but not those treated with either agent alone, showed enhanced release of cytochrome c into the cytoplasm (Fig. 5B) and increased cleavage of poly(ADP-ribose) polymerase (PARP; Fig. 5A). To test whether the combination of E1A and Bcl-2-ASO led to caspase-9 activation, we measured caspase-9 activity in lysates from 2774 cells and found that caspase-9 activity was increased 2-fold in those cells compared with untreated cells at 30 hours after infection (Fig. 5C). Further, the addition of a caspase-9 inhibitor Z-LEHD-FMK reduced cell death induced by Ad.E1A and Bcl-2-ASO to a significantly greater extent than did Ad.E1A plus SCO (Fig. 5D). These findings indicate that the enhanced apoptosis resulting from the combination of E1A plus Bcl-2-ASO is a consequence of cytochrome c release (Fig. 5B) and subsequently caspase-9 activation (Fig. 5C), suggesting that Bcl-2-ASO enhances E1A-induced apoptosis at least in part through activating the mitochondrial death pathway.

**Antisense Bcl-2 plus E1A gene therapy prolongs survival in mice with high Bcl-2–expressing ovarian tumor cells.** After confirming that E1A in combination with Bcl-2-ASO reduced cell viability and enhanced apoptosis in vitro, we examined whether combining Bcl-2-ASO with E1A gene therapy would translate into improved survival in a xenograft model of ovarian cancer. In this model, mice were given i.p. injections of human 2774 cells and then treated with PBS, Ad.E1A(+), Ad.E1A(-), Ad.E1A(+) plus SCO, or Ad.E1A(+) plus Bcl-2-ASO. First, we used immunohistochemical analysis of tumor tissues to verify that the tumor cells were being transfected by the Ad.E1A(+) and that the Bcl-2-ASO was effectively down-regulating Bcl-2 in those cells. As expected, E1A protein was expressed only in tumors from mice treated with Ad.E1A(+) (Fig. 6A, c), and tumors from mice treated with Bcl-2-ASO showed reduced Bcl-2 expression (Fig. 6A, e). With regard to survival, mice in the Ad.E1A(+) plus Bcl-2-ASO group survived longer than did mice in the PBS, Ad.E1A(-), or Ad.E1A(+)-only groups (P < 0.001;
Discussion

Our results confirmed that EIA gene therapy is effective in some, but not all, low HER2-expressing ovarian cancers. The novel aspect of our findings is the discovery that the differences in the effectiveness of such therapy depend on the extent of Bcl-2 expression by the ovarian cancer cells: Ovarian cancer cells that expressed high levels of Bcl-2 were more resistant to EIA treatment than were cells with low Bcl-2 expression. This resistance was also observed in the breast cancer cell lines MDA-MB-435 and MDA-MB-468, both of which express high levels of Bcl-2. In the high Bcl-2–expressing cell lines 2774 and HEY, EIA showed an increase in the number of colonies. Bcl-2 has been shown by other investigators to block E1A-induced activation of caspase-8 and cell death in human epithelial cells (24) and to cooperate with E1A to transform primary cells in culture (25).

However, transformation of primary cells does not always translate into acceleration of tumor formation in vivo, as EIA did not accelerate tumor growth in vivo. We confirmed the importance of Bcl-2 in EIA therapy in vivo in an ovarian cancer xenograft model in which nude mice with tumors consisting of low HER2-, high Bcl-2–expressing ovarian cancer cells were treated with a combination of Ad.EIA(+) and Bcl-2-ASO. This combination prolonged the survival of these mice over that of those treated with Ad.EIA(+) or Bcl-2-ASO alone. Through these results, we have identified Bcl-2 as a potential molecular marker for predicting the outcome of EIA gene therapy in low HER2-expressing ovarian cancers.

EIA suppresses tumor growth by inducing apoptosis through a variety of mechanisms (13, 15, 26), including the induction of p19ARF, which leads to accumulation and stabilization of p53 and subsequent apoptosis (27). EIA also induces apoptosis through p53-independent mechanisms (15). EIA sensitizes cells to many different stimuli, such as serum starvation (13), ionizing radiation (28), DNA-damaging agents (29), TNF (30), and TRAIL (31), all of which can lead to apoptosis.

Preclinical evidence indicates that EIA, delivered by means of adenoviral vectors, DC-Chol cationic liposomes, and liposome/protamine/DNA systems, can have a therapeutic effect in orthotopic animal models of ovarian, breast, lung, and head and neck cancer (6, 7, 32, 33). Phase I clinical trials of EIA gene therapy have been conducted in patients with metastatic or recurrent breast and ovarian cancers (14) or recurrent breast and head and neck cancer (34). In the first of these trials, i.e., injection of the EIA gene in a cationic lipidosome complex in women with ovarian cancer resulted in transfection of ovarian cancer cells with...
E1A and subsequent down-regulation of HER2 expression, reduction of proliferation, and induction of apoptosis in those cells regardless of how much HER2 they expressed (14). Currently, a phase II clinical trial of E1A gene therapy is under way at M. D. Anderson Cancer Center for patients with platinum-refractory ovarian cancer. Identifying molecular markers that can be used to predict the outcome of E1A gene therapy will improve our ability to select patients with breast or ovarian cancer that will respond to E1A gene therapy.

E1A-induced apoptosis is considered to be the major mechanism of its anticancer effect. However, E1A-induced apoptosis can be inhibited by the adenoviral E1B gene, which prolongs the viability of the host cell by inhibiting apoptosis (13). The observation that E1B protein is similar to the antiapoptotic Bcl-2 protein (20) led us to suggest that like E1B, Bcl-2 overexpression in cells may lead to resistance to E1A gene therapy. Expression of high amounts of Bcl-2 protein by ovarian cancer cells has been associated with resistance to cisplatin (35, 36), implying that Bcl-2 is an important component of resistance to chemotherapy. Biopsy samples from patients with ovarian cancer have shown Bcl-2 expression in 27% to 57% of cases (36, 37). Therefore, targeting Bcl-2 in ovarian cancer may be important in the treatment of ovarian cancer.

In this study, we show in an in vivo model that Bcl-2 participates in E1A-mediated antitumor activity, and we are the first to report that Bcl-2 may be responsible for at least some of the resistance to E1A gene therapy seen in ovarian cancer. The usefulness of Bcl-2 as a molecular marker is not restricted to ovarian cancer as Bcl-2 is overexpressed in many hematopoietic malignancies (B-cell chronic lymphocytic leukemia, acute myeloid leukemia and multiple myeloma) and in solid tumors of the breast, bowel, skin, and lung (38).

Figure 5. Antisense Bcl-2 enhances the release of cytochrome c from mitochondria and activates caspase-9 to lead to E1A-induced cell death. A, cells were untreated (Control), treated with Ad.E1A(−) or Ad.E1A(+) for 2 days, or treated with 150 nmol/L of a scrambled oligonucleotide or 150 nmol/L Bcl-2-ASO for 1 day, followed by Ad.E1A(−) or Ad.E1A(+) for 30 hours. Sixty micrograms of whole cell lysates was fractionated on a 13.5% SDS gel and blotted for cleavage of PARP and caspase-8. Actin was used as a loading control. B, cells were treated as indicated in (A) and mitochondrial/cytoplasmic fractionation was done, after which 15 μg of protein was loaded from each fraction and examined for cytochrome c expression by Western blotting. C, cells were treated as indicated in (A) and caspase-9 activity was assayed at 30 hours after Ad.E1A(+) infection. D, cells were treated as indicated in (A), treated or not treated with Z-LEHD-FMK, and then stained with trypan blue for cell viability. The numbers of live and dead cells were counted in four areas under the microscope. Fewer dead cells were present in the Bcl-2-ASO + E1A + caspase-9 inhibitor condition compared with the SCO or E1A conditions.
The usefulness of Bcl-2-ASO therapy for suppressing the expression of Bcl-2 has been well established in preclinical studies (39, 40). In combination with chemotherapy, antisense Bcl-2 therapy has also shown promising antitumor effects in breast cancer (41), lymphoma (39), melanoma (42), prostate (43), small cell lung cancer (44), and chronic myeloid leukemia (45). Bcl-2-ASO has also been used in combination with an antisense construct to MDR-1 to enhance the activity of doxorubicin in multidrug-resistant ovarian and breast cancer cell lines (46). The present study, which evaluated the combination of Bcl-2-ASO used with Ad.E1A(+) in a xenograft ovarian cancer model, provides clear evidence of Bcl-2 down-regulation in vivo and in vitro and apoptosis enhancement in vitro.

Release of cytochrome c into the cytoplasm is a critical event in apoptosis. Because Bcl-2, which is located in the outer membrane of mitochondria, is known to inhibit the release of cytochrome c (47), we hypothesized that antisense Bcl-2 would enhance E1A-induced apoptosis by activating caspase-9 via cytochrome c release. We showed that treating cells expressing high Bcl-2 with antisense Bcl-2 plus E1A led to increased apoptosis. This increase in apoptosis resulted from increased release of cytochrome c into the cytoplasm, with a corresponding decrease in the mitochondria, and from activation of the intrinsic pathway involving caspase-9 activation. These findings are similar to those of another study in which the combination of TRAIL and E1A led to sensitization of cancer cells to TRAIL-induced apoptosis by E1A through enhancement of caspase-8, caspase-9, and caspase-3 activation (16). E1A is known to induce caspase-8 activation and to release cytochrome c from the mitochondria in human epithelial cells, and these effects are inhibited by Bcl-2 (24). In our study, we observed caspase-8 cleavage in E1A-treated cells, but we did not observe enhancement of caspase-8 activation when cells were treated with E1A and Bcl-2-ASO compared with cells treated with E1A only.

Our in vitro finding that Ad.E1A(+) was more cytotoxic than Ad.E1A(−) to low HER2-, high Bcl-2–expressing ovarian cancer cells seems to contradict a previous finding that Ad.E1A(+) had no therapeutic effect in vivo (6). However, this cytotoxic effect was limited and transient; we saw it only in an in vitro trypan blue exclusion assay, not in terms of colony formation (Fig. 1) or mouse survival (Figs. 2B and 6B). In fact, we observed a 2-fold increase in survival rate of nude mice injected with Ad.E1A(−), the control virus (Fig. 6B). Although nude mice lack T cells, their innate immune system is intact and they have functional macrophages and natural killer (NK) cells; thus, these mice are not completely immunodeficient. Repeated administration of recombinant viruses is known to provoke immune responses (48). Moreover, NK cells are known to affect adenovirus-mediated gene therapy; the adenovirus stimulates NK cells, which nonspecifically augment antitumor immunity (49). Peritoneal NK cells are involved in a bystander effect in mice treated with adenoviruses; in that study, a replication-deficient adenovirus without a cytomegalovirus (CMV) promoter and without a transgene expression cassette was shown to recruit and activate peritoneal NK cells (50). Collectively, these findings may explain some of the nonspecific effects we noted in the present preclinical animal study.

Our results also suggest that differences in Bcl-2 levels could explain, at least in part, the heterogeneity in the response of cancer cells to E1A gene therapy. Using Bcl-2 as a molecular marker may also help in appropriately identifying patients with ovarian cancer who would benefit from combination E1A–antisense Bcl-2 therapy.

In summary, we showed here that the success of E1A gene therapy depends, at least in part, on Bcl-2 levels. In our animal model, when Bcl-2 levels in tumor cells were low owing to suppression with Bcl-2-ASO, E1A gene therapy could suppress tumor growth and prolong survival; however, when Bcl-2 levels were intrinsically high, the tumors were resistant to E1A gene therapy (Fig. 6B). Use of Bcl-2 as a molecular marker with which to predict the outcome of E1A gene therapy could greatly benefit patients with ovarian cancer, a cancer that still carries a
particularly poor prognosis. Our next step is to evaluate the ability of Bcl-2 levels to predict response to EIA gene therapy in an ongoing phase II clinical study of EIA gene therapy for ovarian cancer. Future plans include construction of an improved version of the pEIA plasmid in which the antisense Bcl-2 is inserted under the control of another CMV promoter along with the EIA gene. We are hopeful that this approach will lead to the development of more powerful therapies for patients with EIA-resistant tumors.

References

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