In Vivo Cytotoxicity of Ovarian Cancer Cells through Tumor-selective Expression of the BAX Gene

Yu-Tzu Tai, Thomas Strobel, Donald Kufe, and Stephen A. Cannistra

ABSTRACT

The BAX proapoptotic protein is capable of inducing cell death either directly, through its effects on mitochondrial function, or indirectly, by lowering the apoptotic threshold in response to certain chemotherapy agents. In this study, we tested the hypothesis that selective expression of BAX in human ovarian cancer through adenoviral gene transfer might represent a novel approach to eradicating tumor cells in vivo. Two constructs were prepared using replication-deficient adenoviral vectors containing either the cDNA for β-galactosidase (Ad.DF3.GAL) or hemagglutinin (HA)-tagged BAX (Ad.DF3.BAX) under the control of the DF3 promoter. The DF3 promoter was used to confer tumor-specific gene expression in view of its restricted pattern of expression in the majority of human ovarian cancers and its limited expression in normal peritoneal mesothelial cells. In vitro infection of up to seven different epithelial cancer cell lines with Ad.DF3.GAL or Ad.DF3.BAX resulted in expression of either β-galactosidase activity or HA-BAX protein, respectively, which was highly correlated with DF3 levels. Furthermore, infection with Ad.DF3.BAX was capable of highly selective cytotoxicity of DF3-positive ovarian cancer clonogenic cells in vitro. The effect of i.p. administration of Ad.DF3.BAX was also assessed in nude mice inoculated with the DF3-positive 36M2 human ovarian cancer cell line. Expression of either β-galactosidase activity (after Ad.DF3.GAL treatment) or HA-BAX transcripts (after Ad.DF3.BAX treatment) was restricted to tumor tissue in vivo. Importantly, administration of Ad.DF3.BAX on days 2 and 3 after tumor inoculation was capable of eradicating >99% of tumor implants. These results demonstrate the feasibility of tumor selective expression of a proapoptotic protein such as BAX through adenoviral gene transfer.

INTRODUCTION

Advanced epithelial ovarian cancer usually requires treatment with chemotherapy, often with the combination of paclitaxel and a platinum compound such as carboplatin (1). Although such chemotherapy results in tumor cytodestruction in over 70% of patients, drug resistance frequently develops and represents the major cause of death. Because most chemotherapeutic agents mediate their downstream effects through triggering of the apoptotic pathway, we and others have attempted to identify possible abnormalities in ovarian cancer cell apoptosis that might be involved in the development of drug resistance. In this regard, we have found that the BAX proapoptotic protein, a member of the BCL-2 family of apoptotic molecules, is an important determinant of ovarian cancer cell death in response to paclitaxel in vitro. Specifically, stable overexpression of BAX is capable of enhancing paclitaxel cytotoxicity by ~10-fold in the SW626 human ovarian cancer cell line (2, 3). We have recently observed that BAX is underexpressed in tumor specimens from ~40% of patients with ovarian cancer and that underexpression of this protein predicts an inferior response rate to paclitaxel and shortened disease-free survival (4). Taken together, these results suggest that diminished expression of BAX may influence both response to chemotherapy and survival in patients with ovarian cancer.

BAX is localized to the outer mitochondrial membrane by virtue of a COOH-terminal hydrophobic signal anchor, which is typical of several other BCL-2 family members (5). Recent evidence suggests that BAX may partly mediate its apoptotic effects by predisposing to the release of mitochondrial cytochrome c into the cytosol, a critical first step in the activation of an important group of downstream cysteine proteases known as caspases. Specifically, purified BAX alone is capable of inducing cytochrome c release from mitochondrial isolates in vitro (6). In addition to its effects on mitochondrial cytochrome c release, acute overexpression of BAX has been shown to mediate cell death directly, in the absence of an additional toxic stimulus, through a mechanism involving loss of mitochondrial membrane potential (7). Thus, in addition to its ability to lower the apoptotic threshold in response to certain chemotherapeutic agents such as paclitaxel, the BAX protein alone may be capable of mediating cell death through its effects on mitochondrial function, by directly inducing cytochrome c release and/or by leading to damaged mitochondrial membrane integrity.

The fact that BAX expression may predispose to cell death suggests that strategies designed to selectively restore or amplify BAX expression may represent a novel approach for treating human tumors. Although the technique of i.p. gene transfer could be considered a means of introducing the cDNA for BAX into ovarian cancer cells, a theoretical limitation of this approach is nonspecific, high-level, and potentially toxic expression of BAX in normal mesothelial cells, such as those that line the peritoneal cavity. In this regard, the DF3 (MUC1) protein is highly expressed in over three-quarters of epithelial ovarian cancers and is generally weakly or not expressed by normal peritoneal mesothelial cells (8,9). Regulation of DF3 protein expression in epithelial cancer cell lines has been shown to occur in large part at the transcriptional level (10). Accordingly, the DF3 promoter has been previously used to drive the selective transcription of genes such as herpes simplex viral thymidine kinase in DF3-expressing cells. Specifically, i.p. injection of an adenoviral vector containing the herpes simplex viral thymidine kinase gene under the control of the DF3 promoter has been shown to sensitize the DF3-positive MCF-7 breast cancer cell line to the effects of ganciclovir in vivo (11). Here, we describe the construction, expression, and biological activity of a new adenoviral construct containing the BAX gene under the control of the DF3 promoter (Ad.DF3.BAX). We demonstrate that this reagent permits selective expression of BAX in DF3-positive ovarian cancer cell lines and that i.p. BAX gene transfer results in significant tumor cell kill in a nude mouse xenograft model of human ovarian cancer. The implications of this approach for the treatment of human ovarian cancer are discussed.

MATERIALS AND METHODS

Cell Lines. The following human epithelial cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA): SKOV-3, OVCA-3, CAOV-3, and SW626 (ovarian cancer); MCF-7 (breast cancer); DU145 (prostate cancer); and HTB-57 (lung cancer). The 36M2 human epi-
thelial ovarian cancer cell line was developed in our laboratory and has been described previously (12). All lines were grown in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT), 2 mM L-glutamine (BioWhittaker, Inc., Walkersville, MD), 100 units/ml penicillin (BioWhittaker), and 100 μg/ml streptomycin (BioWhittaker).

Constitution of Recombinant Adenovirus. Recombinant adenoviruses containing the cDNA for either β-galactosidase or HA-βGal (Ad.DF3.BGAL or Ad.DF3.BAX, respectively) under the control of the DF3 promoter were prepared with standard homologous recombination techniques using the replication-defective E1/E3-deleted serotype 5 human adenovirus (11, 13). First, a recombinant adenoviral shuttle plasmid containing murine HA-BAX cDNA was constructed by using the previously reported pDF3/LacZ shuttle plasmid, in which the βGAL (LacZ) cDNA is juxtaposed to the DF3 promoter (pDF3; Ref. 11). Specifically, the HA-BAX cDNA was excised from the previously reported pSFFV/HA-BAX plasmid (a kind gift of Dr. Stanley Korsmeyer, Washington University, St. Louis, MO; Ref. 14) and ligated into the pDF3/LacZ shuttle plasmid in place of the LacZ gene. The shuttle plasmid (either pDF3/LacZ to generate control virus or pDF3/HA-BAX) and the adenoviral packaging plasmid pm17 (kindly provided by Dr. Frank Graham, McMaster University, Hamilton, Ontario, Canada) were then cotransfected into the EIA trans-complementing cell line 293 using calcium phosphate precipitation. Transfected cells were maintained until the onset of cellular cytopathic effects. The newly generated recombinant adenovirus (Ad.DF3.BAX) was isolated from a suspension culture of 293 cells that were infected at a multiplicity of infection of 5. Genomic DNA derived from the recombinant adenovirus was analyzed by PCR to verify the presence of HA-BAX in the viral genome. A pair of primers (5′-ACCCATACGACGTCCCAGACTACGC-3′, residing at nucleotides 4038–4061 of the Ada201 sequence) was used to amplify a 567-bp viral vector-specific DNA fragment. Verifiable recombinant adenovirus was expanded within 293 cells and purified by double CsCl gradient ultracentrifugation. The purified adenoviruses were titrated with the 293 cell line using plaque assay techniques for direct determination of viral plaque-forming units. The purified virus was aliquoted in Ad buffer (100 mM Tris-10% glycerol) and stored at –80°C until use. For viral infections in vitro, exponentially growing cells were incubated in the presence of virus (37°C for 2 h) at a variety of MOIs, washed, and transferred to fresh medium for the duration of the experiment. For some experiments, the shuttle plasmid pCMV/LacZ was used to generate the Ad.CMV.βGal recombinant adenovirus in which LacZ expression is driven by the constitutively activated CMV promoter (11).

Estimation of Adenoviral Infection Efficiency. Either cells growing in monolayers or samples from frozen tissue sections (8 μm thick) were washed with PBS and fixed with 0.5% glutaraldehyde (Sigma) for 15 min at room temperature. After three washings for 15 min each in PBS containing 1 mM MgCl₂, cells were stained in X-Gal solution composed of 5 mM K₃[Fe(CN)₆], 5 mM K₄[Fe(CN)₆], 1 mM MgCl₂, and 1 mg/ml X-Gal (Life Technologies Inc., Gaithersburg, MD) for 18 h at 37°C. Infection efficiency was calculated as number of blue cells in a total of 200 cells counted. To assess β-galactosidase activity in the whole mount of peritoneal tissues, tissues were first fixed in 0.2% glutaraldehyde, 0.1 M PBS, 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% NP40 for 1 h, followed by washing for 1 h in the same solution without glutaraldehyde. Tissues were then incubated in X-Gal solution as described above.

Indirect Immunofluorescence Analysis. DF3 expression in epithelial cancer cell lines was determined by flow cytometric analysis. Cells (0.5 × 10⁶ per sample) were treated with either control murine monoclonal antibody (D144, nonreactive IgG1, 10 μg/ml; Ref. 12) or anti-DF3 murine antibody (nonreactive IgG1, 10 μg/ml; Ref. 11) for 30 min at 4°C, followed by two washings and labeling with fluorescein-conjugated goat antimouse immunoglobulin (FITC; Tago, Burlingame, CA) for 30 min at 4°C. After two additional washes, the cells were analyzed using a Coulter Epics C flow cytometer (Coulter, Hialeah, FL).

Immunoblot Analysis. The expression of HA-BAX was evaluated using immunoblot analysis as described previously (2). Antibodies used for immunoblotting were as follows: murine anti-HA monoclonal antibody (1:2000 dilution, clone 12CA5; Boehringer Mannheim, Indianapolis, IN); murine antithetaumin α-tubulin antibody (1:2000 dilution, Sigma); and goat antimouse immunoglobulin conjugated to horseradish peroxidase (1:3000 dilution; Caltag Laboratories, South San Francisco, CA). Protein bands were visualized using enhanced chemiluminescence (ECL; Amersham Corp., Arlington Heights, IL).

Clonogenic Cell Assay. Determination of colony growth in soft agar was performed to assess the effects of Ad.DF3.BAX on clonogenic cell survival. Cells were first plated at a density of 2 × 10³ cells per well in six-well plates 24 h prior to virus infection. Virus infections were performed as described above at MOIs from 0 to 2000 with either Ad.DF3.BAX or the Ad.DF3.βGAL control. After infection, cells were trypsinized and resuspended in 20% FCS/Iscoy’s modified DMEM (Sigma). A bottom layer of 0.5 ml of Iscoye-20% FCS containing 0.5% agar was generated in 24-well plates. After the bottom layer was solidified, 2000–5000 cells per well were seeded in 0.5 ml of the same medium containing 0.3% agar. Quadruplicate determinations were assessed for each viral dose used. Cells were incubated for 14 days at 37°C in 5% CO₂, after which colonies with >30 cells were quantitated in an inverted microscope. The percentage of specific killing was determined as follows: 100 × [(no. of colonies in Ad.DF3.βGAL-infected cells) – (no. of colonies in Ad.DF3.BAX-infected cells)]/(no. of colonies in Ad.DF3.βGAL-infected cells). The use of Ad.DF3.βGAL at MOIs of up to 2000 resulted in <5% cytotoxicity under these assay conditions (compared to the use of diluent alone).

In Vivo Gene Transfer in Nude Mouse Xenografts. The use of 36M2 human ovarian cancer cells in a nude mouse xenograft model of intraabdominal ovarian cancer has been described previously (12). In brief, 4-week-old female athymic nude mice [Tac:Cr:(Ncr)-nuBR; Taconic, Germantown, NY] were irradiated with 300 rad, followed 24 h later by i.p. tumor inoculation with 10 × 10⁶–20 × 10⁶ 36M2 cells in the exponential growth phase of culture. Easily quantifiable tumor implants were visible 3–5 weeks later, depending upon the needs of the experiment. Two different experimental designs were used in this study. First, to evaluate the specificity of gene expression, we exposed animals to Ad.DF3.βGAL at a time when they would be expected to harbor visible intraabdominal tumor nodules, thereby permitting gross assessment of β-galactosidase activity. For this purpose, mice were inoculated i.p. with 20 × 10⁶ 36M2 cells, followed −3 weeks later (at a time when gross nodules are apparent) by i.p. injection with Ad.DF3.βGAL (MOI of 200, 4 × 10⁶ pfu in 0.5 ml of PBS/Ad buffer) on days 23 and 24. The mice were sacrificed on day 25 for whole peritoneal mount and frozen section analysis of tumor implants by X-Gal staining, as described. A similar design was used to assess the expression of HA-BAX in vivo after exposure to Ad.DF3.BAX, with the exception that tissues were collected on day 25 for preparation of total cellular RNA and subsequent RT-PCR analysis (4). The primer pair for HA-BAX is detailed above. The primer pair for β-actin used as a control to assess the quality of the RNA preparation as well as the RT-PCR analysis follows: upper primer, 5′-TCACCACACGTGCCCCAT-3′; lower primer, 5′-GCTTCTGGGTGGAGCATGAT-3′.

A different experimental design was used to assess tumor-specific Ad.DF3.BAX treatment. Irradiated mice (n = 12 per group) were inoculated with 15 × 10⁶ 36M2 cells on day 0, followed by treatment with PBS/Ad buffer alone (n = 11), Ad.DF3.βGAL (n = 11), or Ad.DF3.BAX (n = 12) on days 2 and 3. Viral dose was a MOI of 200 (3 × 10⁶ pfu) in 0.5 ml of PBS/Ad buffer (11). This design assured that a microscopic tumor-burden would be present at the time of viral exposure. Mice were observed for activity and weight over a total of five weeks, after which they were sacrificed to quantitate i.p. nodule formation. Data were expressed as mean ± SE of tumor nodules per treatment group. Significance levels for comparison of differences were determined using the two-sided Student’s t test for unpaired samples.

RESULTS

The Ad.DF3.BAX Adenoviral Vector Demonstrates Selective Expression and Cytotoxicity in DF3-positive Ovarian Cancer Cell Lines in Vitro. Two viral constructs were prepared for this study as described in “Materials and Methods.” The control construct contains the β-galactosidase cDNA driven by the DF3 promoter (Ad.DF3.βGAL) and was used to determine both specificity of expression as well as infection efficiency. The experimental construct...
contains the HA-BAX cDNA driven by the DF3 promoter and was assessed for its ability to induce HA-BAX expression and cytotoxicity of DF3-positive cell lines in vitro. A total of seven human epithelial cancer cell lines (five ovarian, one breast, and one prostate) were infected with Ad.DF3.BGAL (2-h exposure at 37°C, MOI of 200), followed by washing, resuspension in fresh medium, and incubation at 37°C for a total of 48 h. An MOI of 200 was chosen because it provided maximum β-galactosidase expression with minimal intrinsic toxicity of the cell lines used in these experiments. X-Gal staining was assessed at day 3, with the results expressed in Table 1 as a function of surface DF3 expression for each line tested. β-Galactosidase activity was easily detected in each of the DF3-positive cells lines and was generally related to the amount of surface DF3 present. The strongest expression was observed in 36M2 ovarian cancer cells, demonstrating 98% β-galactosidase activity, in association with 91% of cells expressing surface DF3. Weaker expression was observed in SKOV-3 cells, which showed 95% β-galactosidase activity (36M2, SKOV-3, OVCAR-3, and MCF-7) and the SW626 weakly DF3-positive line (17%) were used. As shown in Fig. 1, HA-BAX expression was observed in each of the four strongly positive lines as early as day 1 after infection, with maximal expression usually observed at day 3. As expected, HA-BAX was not observed in cells infected with the negative control Ad.DF3.BGAL construct. Importantly, SW626 cells failed to express HA-BAX for up to 3 days postinfection. Additional experiments carried out to day 5 also confirmed lack of HA-BAX expression in SW626 cells infected with Ad.DF3.BAX (data not shown). The presence of protein in the SW626 immunoblot was confirmed by stripping the blot and reprobing with antitubulin antibody (Fig. 1). Because SW626 cells are known to express endogenous BAX and can support the expression of HA-BAX cDNA (2), it is unlikely that these negative results are due to rapid degradation of virally introduced HA-BAX protein.

These data demonstrate that the Ad.DF3.BGAL and Ad.DF3.BAX constructs are capable of efficient infection and gene expression in a DF3-restricted fashion in human epithelial cancer cells. To evaluate the biological effects of BAX expression in these cells, a variety of cell lines were treated with either Ad.DF3.BGAL or Ad.DF3.BAX (MOI of 0–2000, 2 h at 37°C), followed by washing and plating in agar culture for assessment of clonogenic cell survival at day 14, as described in “Materials and Methods.” These cell lines were chosen based upon their ability to form colonies in agar culture. Clonogenic cells exposed to Ad.DF3.BGAL demonstrated minimal (<5%) cytotoxicity over this time period, and the percentage specific cytotoxicity of Ad.DF3.BAX was assessed by comparison to this control group. As shown in Table 1, the Ad.DF3.BAX construct resulted in low-level expression of β-galactosidase activity in asso-

![Fig. 1. Expression of HA-BAX protein in cells infected with Ad.DF3.BAX. Subconfluent cells from a variety of epithelial cancer cell lines were treated with either Ad.DF3.BGAL (control) or Ad.DF3.BAX adenovirus at a MOI of 200 for 2 h at 37°C, followed by washing and continued incubation in fresh medium for the indicated time period. The percentages of cells expressing DF3 are listed in Table 1 (SW626 was the only line with low-level DF3 expression). Immunoblot analysis was performed using an anti-HA antibody to specifically detect the tagged BAX species contributed by Ad.DF3.BAX. The presence of HA-BAX was observed at day 1 and continued for up to 3 days in all DF3-positive lines tested. In contrast, SW626 cells did not express appreciable levels of HA-BAX. The SW626 blots were stripped and reprobed with murine antihuman α-tubulin antibody to ensure adequate protein quality and equivalency of loading.](image-url)
shown in Fig. 2, treatment with Ad.DF3.BAX over a broad range of MOI resulted in minimal cytotoxicity of SW626 cells (17% DF3-positive) as well as HTB-57 cells (9% DF3-positive). HTB-57 human lung carcinoma cells were used in addition to SW626 cells in these experiments because this line grew well in agar culture and also demonstrated low-level (<10%) DF3 expression. In addition, HTB-57 cells are capable of being infected by recombinant adenovirus, as demonstrated by 85% X-Gal staining after infection with Ad.CMV.βGAL (data not shown). In contrast, the 36M2 human ovarian cancer line, which strongly expressed DF3 (91% positive; Table 1), demonstrated significant (~80%) cytotoxicity after exposure to Ad.DF3.BAX at a MOI of 100 and almost 100% cytotoxicity at a viral concentration of a MOI of 200. The CAOV-3 and SKOV-3 ovarian cancer cell lines, which express lower levels of DF3, demonstrated intermediate level cytotoxicity in response to viral exposure. Specifically, levels of killing observed at a MOI of 500 for CAOV-3 and SKOV-3 cells were 16 and 48%, respectively, compared to 99% for 36M2 cells (Fig. 2). Interestingly, at very high concentrations of virus in the MOI of 2000 range, killing of CAOV-3 and SKOV-3 cells increased to 71 and 85%, respectively, again suggesting that the DF3 promoter may be active in a greater fraction of cells than predicted by the results of flow cytometry (Table 1). It is unlikely that killing of CAOV-3 and SKOV-3 at a MOI of 2000 represents nonspecific expression of BAX in a DF3-negative population of cells because significant cytotoxicity was not observed in SW626 and HTB-57 cells exposed to an equivalent viral concentration (Fig. 2).

l.p. Administration of Ad.DF3.BAX Selectively Eradicates Human Ovarian Cancer Cells in Nude Mouse Xenografts. To assess the in vivo expression of Ad.DF3.βGAL and Ad.DF3.BAX, nude mice were inoculated with 20 × 10⁶ 36M2 cells and allowed to live for 3 weeks prior to i.p. injection of virus, to permit the growth of macroscopic peritoneal tumor implants suitable for X-Gal staining and RNA extraction. 36M2 human ovarian cancer cells were chosen for these studies based upon their high level of DF3 expression, their sensitivity to viral infection as demonstrated above, and their ability to grow well in nude mice (12). Additionally, because the human DF3 promoter is also capable of being activated in DF3-positive murine lineage cells (15), the nude mouse model provides an acceptable method for assessing whether Ad.DF3.BAX is significantly expressed in murine peritoneal mesothelium, for instance. After 3 weeks, the mice received i.p. treatment with either PBS/Ad buffer alone, Ad.DF3.βGAL, or Ad.DF3.BAX in a total volume of 0.5 ml of PBS at a MOI of 200 (4 × 10⁷ pfu) daily for 2 consecutive days (days 23 and 24). The mice were sacrificed on day 25, followed by either X-Gal staining of a whole-mount preparation of the peritoneal surface (Ad.DF3.βGAL group) or isolation of RNA from a variety of tissue sites for the purpose of detecting the virally encoded HA-BAX transcript (Ad.DF3.BAX group). As shown in Fig. 3A, mice treated with Ad.DF3.βGAL demonstrated specific and efficient expression of β-galactosidase activity in macroscopic tumor implants, as opposed to peritoneal mesothelial cells (represented as blue implants after X-Gal staining in the figure). No staining was observed for implants in mice treated with diluent (PBS) alone. Histological examination of tissue sections of tumor stained for X-Gal confirmed the tumor-specific nature of β-galactosidase expression (i.e., none of the mesothelial

Fig. 2. Effects of Ad.DF3.BAX on clonogenic survival of epithelial cancer cells. Cells were treated with either Ad.DF3.βGAL (control) or Ad.DF3.BAX over a broad range of MOIs, followed by washing and plating in agar culture for assessment of day 14 colony formation, as described in “Materials and Methods.” Mean colony numbers were derived from quantitation of quadruplicate wells for each MOI. Background cytotoxicity observed with Ad.DF3.βGAL was <5% at MOIs of up to 2000. Data points, percentage specific cytotoxicity of the Ad.DF3.BAX construct (compared to colony formation in the Ad.DF3.βGAL group), as defined in “Materials and Methods.” ▲, 36M2; ■, CAOV-3; ●, SKOV-3; △, HTB-57; □, SW626.

Fig. 3. Tumor-specific expression of Ad.DF3.βGAL and Ad.DF3.BAX in vivo. Nude mice were inoculated i.p. with 36M2 cells and allowed to form i.p. implants over a 3-week period, as described in “Materials and Methods.” Either Ad.DF3.βGAL or Ad.DF3.BAX was then injected on days 23 and 24 (4 × 10⁷ pfu each dose), followed by sacrifice on day 25. A, magnified view (×7.5) of the peritoneal surface stained with X-Gal to assess β-galactosidase activity (red). B, RT-PCR analysis of total cellular RNA obtained from normal tissue (lung and mesothelium) and tumor implants. Only tissue from tumor implants contained HA-BAX transcripts. The HA-BAX control was obtained from RNA isolated from an SW626 cell line stably transfected with HA-BAX cDNA (2).
BAX GENE TRANSFER IN OVARIAN CANCER

cells stained blue) and demonstrated that only the most superficial layers of the tumor surface were involved in this process (data not shown). This is consistent with the expectation that deeper levels of tumor implants might not be infected by virus administered at a time when macroscopic tumor is present. Expression of the HA-BAX gene transferred by the Ad.DF3.BAX adenovirus was assessed by performing RT-PCR using primers specific for the 5’ HA tag, as described. Primers for β-actin were also used to assure the quality of the RNA preparation and RT-PCR. As shown in Fig. 3B, HA-BAX transcripts were detected only in tumor tissue obtained from Ad.DF3.BAX-treated mice, as opposed to grossly uninvolved areas of peritoneum or tissue outside of the abdominal cavity such as lung. As expected, transcripts were not detected in tumor implants obtained from animals treated with diluent alone.

These data demonstrated that both Ad.DF3.βGAL and Ad.DF3.BAX are capable of being specifically expressed in tumor tissue present within the peritoneal cavity at a MOI of 200 delivered daily for 2 days. Accordingly, the cytotoxicity of Ad.DF3.BAX was next assessed in vivo, specifically at a time when tumor was microscopic so as to maximize the efficiency of viral gene transfer. 36M2 cells (15 × 10⁶) were injected i.p. at day 0, followed by i.p. treatment on days 2 and 3 with either PBS/Ad buffer alone (n = 10 mice), Ad.DF3.βGAL (n = 10 mice), or Ad.DF3.BAX (n = 12 mice) at a MOI of 200 (3 × 10⁹ pfu). The number of tumor implants was assessed after a total of 5 weeks, which we have previously determined to be optimal for quantitation of tumor implant formation in this model. Tumor nodule numbers in diluent-, Ad.DF3.βGAL-, and Ad.DF3.BAX-treated mice were 131 ± 33, 119 ± 30, and 1 ± 1, respectively (mean ± SE), demonstrating ~99% cytotoxicity of the Ad.DF3.BAX construct in this system (difference between the Ad.DF3.BAX- and Ad.DF3.βGAL-treated groups was significant at the level of P < 0.0001). The minor difference between diluent and Ad.DF3.βGAL-treated animals was of borderline significance (P = 0.061). The typical appearance of tumor implants in the three treatment groups is shown in Fig. 4. No differences were observed in mouse activity or weight or in the microscopic appearance of the liver or peritoneal surface, in any of the treatment groups over the duration of the experiment (data not shown).

DISCUSSION

This study demonstrates the feasibility of expressing a proapoptotic protein such as BAX through adenoviral gene transfer at levels sufficient to result in selective death of ovarian cancer cells contained within the abdominal cavity. The selectivity of BAX expression was achieved through the use of the DF3 promoter and is dependent upon high-level expression of DF3 in cancer cells, as opposed to normal peritoneal mesothelium. The use of multiple cell lines with varying degrees of DF3 expression confirms the DF3-specific nature of this effect in vitro and suggests that the observed specificity is not cell line dependent. Importantly, the Ad.DF3.BAX construct was capable of inducing cell death through acute overexpression of an exogenously introduced BAX gene, despite the known presence of endogenous BAX present within many types of epithelial tumor cells (2, 16, 17). Therefore, this approach may be particularly relevant for diseases in which BAX is underexpressed or is functionally impaired, such as in some ovarian, breast, and colon cancers, as well as in certain human leukemias (4, 18–20). Lower expression of BAX in human ovarian and breast cancer tissue has been associated with inferior response to chemotherapy, as well as shortened disease-free survival (in patients with ovarian cancer; Refs. 4 and 18). In addition, a murine model of choroid plexus tumor development has recently been used to demonstrate that loss of BAX is associated with enhanced tumor develop-

Fig. 4. In vivo effects of Ad. DF3. BAX on human ovarian cancer cell survival in nude mice. Nude mice were inoculated i.p. with 36M2 cells, as described in “Materials and Methods,” and then treated i.p. on days 2 and 3 with either diluent (0.5 ml PBS, n = 10; A), Ad.DF3.βGAL (3 × 10⁹ pfu, n = 10; B), or Ad.DF3.BAX (3 × 10⁹ pfu, n = 12; C). The mice were sacrificed after a total of 5 weeks to permit adequate implant quantitation. Tumor nodule numbers in diluent-, Ad.DF3.βGAL-, and Ad.DF3.BAX-treated mice were 131 ± 33, 119 ± 30, and 1 ± 1, respectively (mean ± SE).

2125
ment and shortened animal survival (21). Taken together, these observations suggest that BAX may act as a tumor suppressor gene and that loss of BAX function may result in a tumor survival advantage and/or a diminished response to chemotherapy, either of which may translate into shortened patient survival. Thus, the design of reagents capable of selectively restoring the expression of a proapoptotic protein such as BAX may represent a logical first step in eventually circumventing this problem, resulting in either direct tumor cell death or sensitization of tumor to the effects of chemotherapy. Regarding this last point, the reagent developed for our studies (Ad.DF3.BAX) was extremely efficient at inducing cell death by itself, and it was, therefore, not possible to determine whether BAX gene transfer could overcome chemoresistance to a drug such as paclitaxel, for instance. On the basis of our earlier work using stable BAX transfectants of the SW626 line (2, 3), however, we would expect that BAX gene transfer might also result in enhanced chemotherapy-induced cell death under some circumstances.

Several technical difficulties inherent in adenoviral gene transfer will undoubtedly limit the effectiveness of this and similar approaches in the treatment of ovarian cancer. Although the use of the DF3 promoter has permitted high-level selective expression of BAX in human tumor cells in a murine xenograft model, it is possible that clinically significant, nonspecific gene expression might occur in the human peritoneal cavity and translate into prohibitive toxicity at viral doses required to obtain a tumoricidal effect. Also, heterogeneity of tumor DF3 expression might result in the selection of cells with low-level DF3 promoter activity after repeated exposure to a reagent such as Ad.DF3.BAX. Another well-recognized problem is the immunogenicity of adenoviral proteins, which poses a limitation for repeated i.p. application of this virus to humans. Furthermore, the virus would be expected to infect only the first few layers of cells covering the surface of a gross peritoneal implant. In this regard, it is important to note that the impressive degree of in vivo cytotoxicity of Ad.DF3.BAX in our animal model was observed when the tumor burden was low (microscopic) and when its location was truly superficial, improving the chances of observing a biological effect (Fig. 4).

In contrast, patients with ovarian cancer usually present with bulky tumor masses involving the upper abdomen as well as para-aortic lymph nodes. Even if the tumor can be surgically debulked and chemically cytoreduced to a “microscopic” state, it is often present below the peritoneal mesothelial lining by virtue of its ability to invade into the submesothelial space. Consequently, it may be anticipated that bulky tumor masses that microscopically invade below the peritoneal mesothelium and that also metastasize to local lymph nodes pose serious physical limitations to the effectiveness of this technique. Finally, i.p. distribution of agents such as virus particles may be heterogeneous, with some areas of tumor remaining inaccessible to the virus. Therefore, although the idea of i.p. gene therapy for ovarian cancer at first glance appears reasonable, in many respects this disease is not truly a surface tumor, and the degree of benefit to be achieved with currently available viral vectors remains to be determined. Despite these recognized limitations, our study establishes proof of principle regarding the potential value of targeted expression of BAX in human tumor cells and should form the basis for further investigation of this approach in preclinical models of ovarian cancer.

ACKNOWLEDGMENTS

We thank Dr. Ling Chen for his advice during construction of the Ad.DF3.BAX adenoviral vector.

REFERENCES

In Vivo Cytotoxicity of Ovarian Cancer Cells through Tumor-selective Expression of the BAX Gene

Yu-Tzu Tai, Thomas Strobel, Donald Kufe, et al.


Updated version  Access the most recent version of this article at:  
http://cancerres.aacrjournals.org/content/59/9/2121

Cited articles  This article cites 20 articles, 12 of which you can access for free at:  
http://cancerres.aacrjournals.org/content/59/9/2121.full.html#ref-list-1

Citing articles  This article has been cited by 12 HighWire-hosted articles. Access the articles at:  
/content/59/9/2121.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.