Use of the Probasin Promoter ARR2PB to Express Bax in Androgen Receptor-Positive Prostate Cancer Cells

Francesca Andriani, Bicheng Nan, Jiang Yu, Xiaoying Li, Nancy L. Weigel, Michael J. McPhaul, Susan Kasper, Shunsuke Kagawa, Bingliang Fang, Robert J. Matusik, Larry Denner, Marco Marcelli

Background: Adenovirus-mediated overexpression of the apoptosis-inducing protein Bax can induce apoptosis in prostate cancer cell lines. Constitutive overexpression of Bax could result in unwanted apoptosis in every site of accidental Bax accumulation in vivo. Therefore, we developed an adenoviral construct (Av-ARR2PB-Bax) in which the probasin promoter, modified to contain two androgen response elements, drives Bax expression. This promoter would be expected to limit expression of Bax to cells expressing the androgen receptor. Methods: A variety of androgen receptor (AR)-positive and -negative cell lines of prostatic or nonprostatic origin were infected with Av-ARR2PB-Bax or a control virus, Av-ARR2PB-CAT, in which the same promoter drives expression of the chloramphenicol acetyl transferase-reporter gene. Bax expression and apoptosis in vitro were assessed by western blot analysis. Tumor size and apoptosis in vivo were assessed after four weekly injections of Av-ARR2PB-Bax or Av-ARR2PB-CAT into subcutaneous LNCaP xenografts growing in uncastrated male mice. All statistical tests were two-sided. Results: Bax was overexpressed in an androgen-dependent way in AR-positive cell lines of prostatic origin but not in AR-positive cells of nonprostatic origin or in AR-negative cell lines of either prostatic or nonprostatic origin. The androgen dihydrotestosterone activated apoptosis in LNCaP cells infected with Av-ARR2PB-Bax but not in those infected with Av-ARR2PB-CAT. Av-ARR2PB-Bax injected LNCaP xenograft tumors decreased in tumor size from 34.1 mm^3 (95% confidence interval [CI] = 25.1 mm^3 to 43.1 mm^3) to 24.6 mm^3 (95% CI = -2.5 mm^3 to 51.7 mm^3), but the difference was not statistically significant (P = .5). Tumors injected with Av-ARR2PB-CAT increased in size, from 28.9 mm^3 (95% CI = 12.7 mm^3 to 45.1 mm^3) to 206 mm^3 (95% CI = 122 mm^3 to 290 mm^3) (P = .002) and contained statistically significant more apoptotic cells (23.3% [95% CI = 21.1% to 25.6%] versus 9.5% [95% CI = 8.0% to 11.1%]) (P<.001). Conclusions: Av-ARR2PB-Bax induces androgen-dependent therapeutic apoptosis in vitro and in vivo by activating apoptosis in AR-positive cells derived specifically from prostatic epithelium and does not affect nonprostatic cells. [J Natl Cancer Inst 2001;93:1314-24]

In previous studies (1–5), we have investigated the feasibility of manipulating molecules of the apoptotic pathway for therapeutic purposes in experimental models of prostate cancer. One such molecule is Bax, which is a proapoptotic member of the Bcl-2 family of proteins. We found that adenovirus-mediated Bax overexpression induced apoptosis within 24 hours after infection in a number of prostate cancer cell lines by activating the mitochondrial pathway of apoptosis (6). Furthermore, after three intratumoral injections of the adenovirus-mediated Bax overexpression system, xenograft PC-3 cell tumors grown in nude mice regressed 25% in size, which corresponded to a 90% reduction in tumor size relative to the tumor size in mice given an injection of the control binary system expressing β-galactosidase (6). Thus, adenovirus-mediated overexpression of Bax has proven to be an effective way to induce therapeutic apoptosis in experimental models of prostate or other cancers (7–10).

Although powerful, an adenoviral system that constitutively overexpresses Bax is potentially associated with substantial side effects because it could induce unwanted apoptosis in every site of accidental Bax accumulation. Thus, the development of a system driven by a promoter that is sufficiently powerful to overexpress the therapeutic gene in the prostatic target tissue and sufficiently selective to be active only in prostatic epithelium is mandatory if complementary DNAs (cDNAs) encoding for apoptosis-promoting molecules, such as Bax, are to be used in gene therapy. Adenoviral vectors have been constructed that use promoters that are specific to the prostatic epithelium, such as probasin or prostate-specific antigen (PSA), to specifically overexpress the molecule of interest only in the prostate (11). However, the first-generation probasin (−426/+28-base pair [bp] 5′ upstream of the rat probasin gene) and PSA (650-bp 5′ upstream of the human PSA gene) promoters are generally less powerful than constitutively active viral promoters, and their ability to induce therapeutic effects in target cells is uncertain (11). Other prostate-specific reagents, including the prostate-specific membrane antigen promoter and enhancer (12), have been identified, but their efficacy in vivo by use of adenoviral technology is, at this time, unknown.

To develop an adenoviral vector for targeting prostatic epithelium, we decided to use the third-generation probasin promoter ARR2PB, which was developed by the laboratory of Matusik (13) from the original rat probasin promoter and modified to contain two androgen-responsive regions (ARRs). Transfection of reporter genes driven by ARR2PB was followed by a greater than 200-fold induction of reporter gene activity in an...
androgen-dependent manner in androgen receptor (AR)-positive cell lines derived from prostatic epithelium (13). In addition, the same promoter was highly specific for prostatic epithelium in transgenic mice (13).

For the current study, we hypothesized that adenoviral particles containing the ARR2PB promoter linked to the Bax cDNA may have a number of advantages over previous constructs developed as gene therapies for the treatment of prostate cancer. First, unlike constitutively active promoters, the ARR2PB promoter should be specific to prostatic epithelium. Second, unlike previous prostate-specific promoters, the ARR2PB promoter should be powerful enough to drive adequate overexpression of the therapeutic gene to elicit a prompt therapeutic effect. Third, overexpression should be dihydrotestosterone inducible.

Using a large number of AR-positive or AR-negative cell lines from a variety of tissues, we performed experiments to establish 1) if an adenovirus containing the Bax cDNA driven by the ARR2PB promoter can be used to overexpress the gene of interest uniquely in AR-positive prostatic epithelium and 2) if, in this system, overexpression of Bax is dihydrotestosterone dependent, occurs in AR-positive but androgen-independent cell lines, and is followed by apoptosis in vitro and in vivo.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) and tissue culture medium were from Life Technologies, Inc. (GIBCO BRL), Rockville, MD. Chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Antibodies and other reagents for apoptosis assays have been described previously (1–5). The full-length Bax cDNA containing a hemagglutinin (HA) tag at the amino terminus was a gift from Dr. Stanley Korsmeyer (Harvard University Medical School, Boston, MA). The antibody to detect cleaved caspase-3 was from Cell Signaling Technology (Beverly, MA).

Cell Lines

Several cell lines from different organs were used for these studies. The prostate cancer-derived cell lines LNCaP, PC3, DU-145, and TSU-Prl have been described previously (3). The prostate cancer-derived cell line C4-2 (14), maintained in 80% Dulbecco’s modified Eagle medium (DMEM), low glucose, and 20% Knock’s F12 with 5% heat-inactivated FBS supplemented with insulin (5 μg/mL), triiodothyronine (13.65 μg/mL), atrapertin (5 μg/mL), t-biotin (0.244 μg/mL), and adenosine (25 μg/mL) (all from Sigma Chemical Co.) and 1% penicillin and streptomycin, was purchased from UroCor Inc. (Oklahoma City, OK). The prostatic epithelium-derived M12 cells (15), maintained in RPMI-1640 medium, with 5% FBS and 1% penicillin and streptomycin, were a gift from Dr. Joy L. Ware (Virginia Commonwealth University, Richmond) and Steven Plzyme (University of Washington Medical School, Seattle). PC-3 AR and M12-AR are clones of PC-3 and M12 cells that were stably transfected with an AR cDNA. The AR cDNA plasmid used for stable transfection has been described previously (16). Stable transfectants were essentially generated as described by Marcelli et al. (17) by use of lipofectamine (Life Technologies Inc.) according to the manufacturer’s specifications and were selected with the use of G418 (Life Technologies Inc.) at the final concentration of 400 μg/mL.

The breast cancer-derived MDA-MB-453 (18), MDA-MB-231 (18), and ZR-75 (19) cell lines, all maintained in improved minimum essential medium (IMEM) (Zn2+ option), with 10% FBS, 1% penicillin and streptomycin, were obtained from the American Type Culture Collection (Manassas, VA). Foreskin fibroblast strains 881 (22), 1003 (23), and 1017 (23), all maintained in minimal essential medium (MEM) with 10% FBS and 1% penicillin and streptomycin, have been described previously. Low-passage 293 cells, maintained in IMEM (Zn2+ option), with 10% FBS, 2 mM L-glutamine, and 1% penicillin and streptomycin, were purchased from Microbiex Biosystem (Toronto, ON, Canada).

Preparation of Av-ARR2PB-Bax

Adenoviral particles containing the human Bax cDNA linked to the ARR2PB promoter were prepared as described previously (1). Briefly, a full-length HA-tagged Bax cDNA was subcloned downstream of the ARR2PB promoter on the background of the Bluescript plasmid (KS+) (Stratagene Cloning Systems, San Diego, CA). The ARR2PB-Bax cassette was then digested with the restriction endonucleases XhoI and XhoI and subcloned into the shuttle plasmid pXCJL-1 poly A+, which had been digested with EcoRV and XhoI. The resulting construct was named pXCJL-1-ARR2PB-Bax-poly-A. pXCJL-1-poly-A (from Dr. BaBie Teng, The University of Texas Medical School, Houston) has a PBR322 backbone and contains the human adenovirus type 5 (Ad5) 5′-inverted terminal repeat, the Ad5 origin of replication, the Ad5 encapsidation signal, the E1a enhancer, multiple cloning sites, a polyadenylation signal at the 3′ of the polylinker, and the Ad5 sequence from nucleotide positions 3328–6246, which serves as an homologous recombination fragment.

The recombinant adenovirus Av-ARR2PB-Bax was prepared by cotransfection of the pXCJL-1-ARR2PB-Bax-poly-A and pJM17 (24), which contains a full-length adenoviral genome, into low-passage 293 cells (25) seeded at a density of 2 × 106 cells/dish onto 60-mm culture dishes the day before transfection by the calcium phosphate coprecipitation method (26). Two weeks after transfection, recombinant adenoviral plaques were picked, propagated, and screened for the expression of ARR2PB-Bax sequences by polymerase chain reaction (27). Adenoviral particles (named Av-ARR2PB-Bax) that contained ARR2PB-Bax were purified by a large-scale purification method described previously (2).

Other Adenoviral Constructs

Adenovirus Av-ARR2PB-CAT (Kasper S, Matusik RJ; unpublished data), containing the chloramphenicol acetyl transferase (CAT) reporter gene under the control of the ARR2PB promoter, was used as a control for Av-ARR2PB-Bax. The binary system for the overexpression of Bax consists of two adenoviruses (Ad/PKG/GV16 and Ad/GT-Bax) (9). Ad/PKG/GV16 produces a powerful transcription factor, the GAL4-VP16 fusion protein under the control of the constitutively active phosphoglycerate kinase promoter. Ad/GT-Bax produces Bax under the control of a GAL/TATA minipromoter. Thus, when introduced together, the constitutively produced GAL4-VP16 binds the GAL/TATA minipromoter and drives transcription of Bax.

Experimental Protocol

Two days before adenoviral infection, 1 × 105 cells were seeded in each well of a six-well plate. On the day of the infection, the cells from one well were detached with trypsin and counted. This information was used to infect each cell line at the desired multiplicity of infections (MOIs). Infections were carried out with 500 μL of infection medium (the same medium used for each cell line with 2% FBS and 1% penicillin and streptomycin) in a 5% CO2 incubator at 37°C for 1 hour on a rocker.

Pilot experiments with an adenovirus containing the green fluorescent protein (GFP) cDNA (Av-GFP) under the cytomegalovirus promoter determined that the optimal MOI for most cell lines was 100:1 (data not shown). However, to obtain 100% positive GFP expression, some cells (such as the foreskin fibroblasts, MDA-MB-453 and MDA-MB-231 cells) had to be infected at an MOI of 500:1, and others (such as the PC-3) had to be infected at an MOI of 1000:1. Infection with the binary system was done with the use of a ratio of 2:1 of Ad/GT-Bax to Ad/PKG/GV16 at a final MOI of 100:1. Each experiment was performed in medium with regular FBS (or with charcoal-stripped FBS when indicated). One hour after infection, cells were treated with or without dihydrotestosterone (Seraloids, Newport, RI) at the saturating concentration of 2 nM for 48 hours. In some experiments, increasing concentrations of dihydrotestosterone (range, 0.01–100 nM) were used to treat LNCaP cells growing in regular or charcoal-stripped FBS. In some experiments, the nonmetabolizable androgen, mibolerone (BioMol, Plymouth Meeting, PA), was used at a concentration of 2 nM. In further experiments, LNCaP cells were
stimulated either with 10^{-8} M dihydrotestosterone and increasing concentrations of hydroxyflutamide (10^{-8} to 10^{-5} M) (Schering-Plough Research Institute, Kenilworth, NJ) or with the highest concentration of 10^{-5} M hydroxyflutamide alone. These experiments were carried out in LNCaP cells because these cells carry a mutation of the AR, and functional studies (28–30) have shown that this mutation alters the AR so that hydroxyflutamide becomes a strong agonist.

In most experiments, cells were infected with Av-ARR_{2PB-Bax}, with the end point of quantifying Bax expression by western blot analysis. Uninfected cells, or cells treated with the control adenovirus Av-ARR_{2PB-CAT}, were used as a control in each of these experiments. For the immunodetection of Bax, we used previously published protocols (3,6). Densitometric analysis was used to quantify Bax expression relative to that of β-actin, which was immunodetected in the same sample. Densitometry was done by importing images to a Power Macintosh G3 personal computer by use of the Chemi Doc Documentation System and the Quantity One quantitation software (both from Bio-Rad Laboratories, Hercules, CA). Arbitrary densitometric units of the protein of interest were then corrected for the densitometric units of β-actin. The Bax/β-actin ratio obtained under various experimental conditions was then divided by the value obtained under control conditions to calculate the fold induction of Bax.

In the hydroxyflutamide experiment, LNCaP cells were infected with Av-ARR_{2PB-Bax}, and the end point was quantifying Bax expression by western blot analysis. When this same experiment was performed with the use of Av-ARR_{2PB-CAT}, the end point was measurement of CAT activity. CAT activity was measured as reported previously (31).

Analysis of the Apoptotic Pathway

Two days after infection with Av-ARR_{2PB-Bax} (in the presence or absence of 2 nM dihydrotestosterone) or Av-ARR_{2PB-CAT} (in the presence or absence of 2 nM dihydrotestosterone) and 24 hours after infection with the binary system overexpressing Bax (in the presence or absence of 2 nM dihydrotestosterone), cells were harvested and analyzed for Bax overexpression, procaspase-7 and cleaved caspase-3 expression, cytochrome c subcellular localization, DNA fragmentation factor (DFF) cleavage, DEVDase activity (i.e., the catalytic activity of the effector caspases caspase-3 and -7), and terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL), with the use of the techniques described previously (1–4).

Fig. 1. Androgen receptor (AR) expression in the various cell lines and standard curve of Bax expression in LNCaP cells. The prostatic cell lines used were LNCaP, PC-3, PC-3-AR, TSU-Pr1, DU-145, C4–2, M12, and M12-AR. The breast cancer-derived cell lines used were MDA-MB-453, MDA-MB-231, and ZR-75–1. The melanoma-derived cell line used was IIB-Mel-J (identified as IIB). The pancreatic cancer-derived cell line used was Panc-1. The foreskin fibroblast cell lines used were 1017, 1003, and 881. A) Immunoblot showing the AR status of the cell lines used in the study. B) Standard curve of Bax overexpression obtained in LNCaP cells growing in normal or charcoal-stripped fetal bovine serum. Cells were infected at a multiplicity of infection (MOI) of 100; and were stimulated with the indicated concentrations of dihydrotestosterone (DHT) for 24 hours. Cells were collected and lysates were then subjected to western blot analysis with antibodies for Bax and β-actin. β-Actin was included to demonstrate equivalent protein loading. The protein sizes in kilodaltons (kDa) are indicated.

In Vivo Studies

In vivo studies were approved by the Center of Comparative Medicine of the Baylor College of Medicine. LNCaP cells were used because this is the standard AR-positive and androgen-sensitive cell line. Uncastrated male mice were used because the endogenous testosterone produced by these animals is necessary to sustain growth of xenografted LNCaP cells and to drive ARR_{2PB} promoter activity once the constructs Av-ARR_{2PB-Bax} or Av-ARR_{2PB-CAT} were inoculated inside the tumor. No exogenous androgens were used in these experiments.

LNCaP cells (5 × 10^{6}) were dispersed in RPMI-1640 medium with 20% Matrigel and injected subcutaneously in six 56-day-old male nu/nu mice (Harlan Sprague-Dawley, Indianapolis, IN). Each mouse received two tumors, for a total of 12 tumors. Thirty days later, when tumors averaged 30 mm^3 in size, a treatment regimen was started with weekly injections of 1.9 × 10^{8} plaque-forming units (pfu) for 4 weeks. Six tumors were injected directly with Av-ARR_{2PB-CAT} (the control group) and six with Av-ARR_{2PB-Bax} (the treated group). Each tumor was measured weekly with the use of calipers, and the tumor volume was calculated by the equation $m_2^2 	imes m_1 	imes 0.5236$ (where $m_1$ and $m_2$ are the smallest and largest diameters, respectively) (32). One week after the last inoculation of virus, the mice were killed, their tumors were excised and weighed, and autopsies were performed.

Statistical Analysis

All of the data are expressed as mean ± 95% confidence intervals (CIs), unless otherwise stated. To statistically compare the tumor sizes between the treated and control mice, we averaged the weight of the two tumors in the same mouse and performed a two-tailed paired Student’s $t$ test. In addition, once the tumors were excised, they were analyzed for the presence of TUNEL-positive cells by use of techniques described previously (1). Five hundred cells were scored for TUNEL positivity in each tumor (50 cells in 10 different random fields of the slide). To account for the correlation between tumors grown in the same mouse, we averaged the number of TUNEL-positive cells between the two tumors from the same animal and performed statistical analysis of three treated versus three control mice. The number of TUNEL-positive cells and the size of the tumors in the two groups during the 5 weeks of the study were compared by use of two-tailed paired Student’s $t$ tests. Statistical significance was $P<.05$. 

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RESULTS

ARR2PB-Bax Infection in Prostate Cancer and AR-Positive LNCaP Cells

To determine the phenotype of the cell type in which the ARR2PB promoter overexpresses Bax and whether this overexpression is followed by apoptosis, we used a variety of AR-positive or AR-negative cell lines of prostatic or nonprostatic derivation (Fig. 1, A). Each experiment was carried out by use of Av-ARR2PB-CAT as a negative control and the binary system overexpressing Bax as a positive control.

Infection of LNCaP cells with Av-ARR2PB-Bax at an MOI of 100:1 and stimulation with increasing concentrations of dihydrotestosterone (0.01–100 nM) produced a dose-dependent induction of Bax expression (Fig. 1, B). Bax expression and induction were similar in the presence of normal serum or serum charcoal stripped to remove endogenous hormones (Fig. 1, B). However, peak-inducible Bax expression was obtained with a 10-fold lower concentration of dihydrotestosterone in cells treated with charcoal-stripped serum (Fig. 1, B), suggesting that removal of any endogenous sources of androgens from the culture medium did not affect the ability of Av-ARR2PB-Bax to be induced.

Fig. 2. Bax overexpression in androgen receptor (AR)-positive (LNCaP) or AR-negative (PC-3) prostate cancer cell lines. A) Immunoblot analysis of LNCaP and PC-3 cells after infection with adenoviral vectors (Av) Av-ARR2PB-CAT (lanes 1–4) or Av-ARR2PB-Bax (lanes 6–11). Adenoviral infection with a binary system (Ad/PGK/GV16 and Ad/GT-Bax) (lanes 12–15), which uses a constitutively active promoter unrelated to the ARR2PB promoter, was used as a positive control. Control uninfected cells are shown in lane 5. LNCaP cells were infected at a multiplicity of infection (MOI) of 10:1 (lanes 1 and 2, 6–8, and 12 and 13) or 100:1 (lanes 3 and 4, 9–11, and 14 and 15), whereas PC-3 cells were infected at MOIs of 100:1 (lanes 1 and 2, 6–8, and 12 and 13) or 1000:1 (lanes 3 and 4, 9–11, and 14 and 15). Cells were then stimulated in the absence (lanes 1, 3, 5, 6, 9, 12, and 14) or in the presence (lanes 2, 4, 7, 10, 13, and 15) of 2 nM dihydrotestosterone (DHT) or 2 nM mibolerone (lanes 8 and 11). After 24 hours, cells were collected and lysates were subjected to western blot analysis with antibodies for Bax and β-actin. β-Actin was included to demonstrate equivalent protein loading. The Bax/β-actin ratio was determined by densitometry to quantify the differences in Bax expression relative to β-actin. B) The additional 23-kilodalton (kDa) Bax band detected in cells infected with the Av-ARR2PB-Bax construct is a hemagglutinin (HA)-tagged form of the protein. LNCaP cells were infected with Av-ARR2PB-Bax (lanes 1 and 3) or Ad/PGK/GV16 and Ad/GT-Bax (lanes 2 and 4). Av-ARR2PB-Bax-infected cells were stimulated with 2 nM dihydrotestosterone. Cells were collected and lysates were subjected to western blot analysis by use of an anti-Bax (lanes 1 and 2) or anti-HA antibody (lanes 3 and 4). Bax expression was not detectable in uninfected LNCaP cells by western blot analysis (Fig. 2, A, lane 5). When LNCaP cells were infected with Av-ARR2PB-Bax at an MOI of 10:1, Bax expression was detected only after addition of dihydrotestosterone (compare Fig. 2, A, lanes 6–8). At an MOI of 100:1, Bax expression was detected in untreated cells and could be further induced after the addition of dihydrotestosterone or mibolerone (Fig. 2, A, lanes 9–11). Densitometric analysis of this experiment, in which the level of Bax was normalized to that of β-actin (Bax/β-actin ratio), demonstrated that, at an MOI of 100:1 and in the presence of dihydrotestosterone, Bax expression was increased approximately 1300-fold compared with uninfected LNCaP cells (Fig. 2, A, compare lanes 10 and 5) or with LNCaP cells infected at an MOI of 10:1 but not stimulated with dihydrotestosterone. In addition, Bax expression in cells infected with Av-ARR2PB-Bax was sevenfold higher than that in cells infected with the binary system, which constitutively overexpresses Bax (Fig. 2, A, compare lanes 10 and 15).

Next, we confirmed that the two bands migrating as a doublet in Av-ARR2PB-Bax-infected cells represented a 23-kilodalton (kDa) form of Bax containing the HA tag at its amino terminus and the 21-kDa wild-type form (Fig. 2, B, lane 1). An anti-HA antibody recognized only the higher molecular weight band...
overexpressed in lysates from cells infected with Av-ARR2PB-Bax (Fig. 2, B, lane 3) and no bands in lysates from cells infected with the binary system (Fig. 2, B, lane 4). By contrast, an anti-Bax antibody (Fig. 2, B) recognized both the upper and lower bands of Bax overexpressed in lysates from cells infected with Av-ARR2PB-Bax (Fig. 2, B, lane 1), and the only lower molecular weight band overexpressed in lysates from cells infected with the binary system (Fig. 2, B, lane 2). Thus, two forms of the Bax protein are synthesized in Av-ARR2PB-Bax-infected cells. One form, initiated from the first methionine of the coding region of the construct, incorporated the HA tag. The other, presumably initiated from the first methionine of the Bax cDNA, resulted in a protein that comigrated with that produced in cells infected with the binary system, which does not have an HA tag.

**ARR2PB Infection in AR-Negative Prostate Cancer Cells**

We next determined if Av-ARR2PB-Bax could be expressed in AR-negative cell lines derived from prostatic tissue. Bax expression was not inducible in AR-negative cell lines (Fig. 1, A), such as PC-3 (Fig. 2, A), DU-145, TSU-Pr1, and M12 (data not shown) after infection with Av-ARR2PB-Bax (MOI 100 : 1, except PC-3 where an MOI of 1000 : 1 was used) before or after the addition of dihydrotestosterone. By contrast, Bax overexpression was inducible in the same cell lines after infection with the binary system.

To further examine the relationship between AR and Bax expression in prostate-derived cells, we stably transfected PC-3 and M12 cells with an AR-expression vector and then infected them with Av-ARR2PB-Bax. Dihydrotestosterone could induce Bax expression in PC-3-AR cells that were infected with Av-ARR2PB-Bax by almost threefold (Fig. 3) and in M12-AR cells infected with Av-ARR2PB-Bax by 1.6-fold (Fig. 3). Although infection with the binary system was associated with Bax overexpression in both PC-3-AR and M12-AR, there was no further induction of Bax after the addition of dihydrotestosterone (Fig. 3, compare lanes 4 and 5).

**Av-ARR2PB-Bax Infection in AR-Positive Androgen-Independent Cell Lines**

To determine if Bax can be overexpressed in cells that proliferate in the absence of androgens, we used the AR-positive C4–2 cells. These LNCaP-derived cells, unlike LNCaP cells, have acquired the ability to grow in steroid-depleted medium and in castrated hosts. They have acquired a substantial metastatic potential and represent a model of AR-positive androgen independence (14). According to the Bax/β-actin ratio, Bax expression was induced by approximately 1500-fold (95% CI = 1344 to 1723) in C4–2 cells after infection with Av-ARR2PB-Bax and treatment with dihydrotestosterone (Fig. 3, lanes 2 and 3), confirming that these cells have retained an intact AR-signaling pathway. The degree of Bax induction was 1.1-fold times (95% CI = 0.1063 to 2.094) that observed in cells infected with a similar MOI of the binary system (Fig. 3, compare lanes 3 and 5). No induction of Bax was seen in control cells infected with Av-ARR2PB-CAT (not shown).

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**Fig. 3.** Bax expression in adenovirus-infected androgen receptor (AR)-negative prostate cancer cell lines (PC-3-AR and M12-AR) after stable transfection with an AR-expression plasmid and in an AR-positive, androgen-independent cell line (C4–2). Immunoblot analysis of PC-3-AR, M12-AR, or C4–2 cells after infection with Av-ARR2PB-Bax (lanes 2 and 3). Uninfected cells (lane 1) were used as a negative control and cells infected with an adenoviral binary system (Ad/PGK/GV16 and Ad/GT-Bax) (lanes 4 and 5), which uses a constitutively active promoter unrelated to the ARR2PB promoter, were used as a positive control. Cells were infected at a multiplicity of infection (MOI) of 100 : 1 (except PC-3-AR, which were infected at an MOI of 1000 : 1) and treated in the absence (lanes 1, 2, and 4) or in the presence (lanes 3 and 5) of 2 nM dihydrotestosterone (DHT). After 24 hours, cells were harvested and lysates were subjected to western blot analysis with antibodies for Bax and β-actin. β-Actin was included to demonstrate equivalent protein loading. The protein sizes in kilodaltons (kDa) are indicated.
Infection of Av-ARR<sub>2</sub>PB-Bax in AR-Positive Cell Lines of Nonprostatic Derivation

Experiments were then performed in cell lines derived from other tissues to determine if the expression of Bax driven by the ARR<sub>2</sub>PB promoter was specific to AR-positive prostatic epithelium. We tested AR-positive (MDA-MB-453 [Fig. 4] and ZR-75–1 [not shown]) and AR-negative (MDA-MB-231 [Fig. 4]) breast cancer cell lines and three AR-positive foreskin fibroblast cell lines. One (1017, Fig. 4) of these was from an individual with no known pathologic condition, one (881, Fig. 4) was from an individual with complete androgen insensitivity because of a mutation in the AR DNA-binding domain causing its transcriptional silencing (22), and one (strain 10003, data not shown) was from an individual with a Reifenstein phenotype, which causes a qualitative defect in dihydrotestosterone binding because of a mutation in the AR hormone-binding domain (23).

Infection with Av-ARR<sub>2</sub>PB-Bax and treatment with dihydrotestosterone were not followed by an induction of Bax expression in any of these cell lines (Fig. 4), regardless of AR status. By contrast, Bax overexpression was substantial after each cell line was infected with the binary system, which has a constitutively active promoter.

Infection of Av-ARR<sub>2</sub>PB-Bax in Cell Lines Derived From Other Organs

AR-negative cell lines derived from other tissues, such as melanoma (IIB-MEL-J; data not shown) and pancreatic cancer (Panc-1; data not shown), were used to rule out the possibility that the ARR<sub>2</sub>PB promoter drives gene transcription in cells of nonprostatic derivation. Again, although Bax overexpression was not detected in cells infected with Av-ARR<sub>2</sub>PB, Bax overexpression was substantial in cells infected with the binary system.

Stimulation With Hydroxyflutamide

LNCaP cells contain ARs with a mutation in the steroid-binding domain (Thr 868 changed to Ala) that results in a change in hormone specificity (28). Because this mutant AR can be activated by hydroxyflutamide (33), we tested this drug to determine if it works as an AR agonist in LNCaP cells infected with Av-ARR<sub>2</sub>PB-Bax or Av-ARR<sub>2</sub>PB-CAT. Cells were stimulated overnight with 10<sup>−5</sup> M hydroxyflutamide and then analyzed for Bax expression (when Av-ARR<sub>2</sub>PB-Bax was used) (Fig. 5, A). In other experiments, LNCaP cells were stimulated either with 10<sup>−8</sup> M dihydrotestosterone and increasing concentrations of hydroxyflutamide (10<sup>−8</sup> to 10<sup>−5</sup> M) or with the highest concentration of 10<sup>−5</sup> M hydroxyflutamide alone and then were analyzed for CAT activity (when Av-ARR<sub>2</sub>PB-CAT was used) (Fig. 5, B). There was no induction of Bax expression or CAT activity when hydroxyflutamide was given alone, and hydroxyflutamide was effective in reducing dihydrotestosterone-induced CAT activity when both treatments were given together. These experiments suggest that, under these experimental conditions, OH–flutamide did not work as an AR agonist in LNCaP cells.

**Fig. 4.** Bax expression in adenovirus-infected androgen receptor (AR)-positive cell lines of nonprostatic derivation. Immunoblot analysis of MDA-MB-453, MDA-MB-231, 1017, and 881 cells after infection with Av-ARR<sub>2</sub>PB-Bax (lanes 2 and 3). Uninfected cells (lane 1) were used as a negative control, and cells infected with an adenoviral binary system (Ad/PKG/GV16 and Ad/GT-Bax) (lanes 4 and 5), which uses a constitutively active promoter unrelated to the ARR<sub>2</sub>PB promoter, were used as a positive control. Cells were infected at a multiplicity of infection (MOI) of 500:1 and treated in the absence (lanes 1, 2, and 4) or in the presence (lanes 3 and 5) of 2 nM dihydrotestosterone (DHT). After 24 hours, cells were collected and lysates were subjected to western blot analysis with antibodies for Bax and β-actin. β-Actin was included to demonstrate equivalent protein loading. The protein sizes in kilodaltons (kDa) are indicated.
We next investigated whether ARR2PB-induced Bax overexpression was associated with apoptosis in LNCaP cells. We first measured apoptosis in LNCaP cells 48 hours after infection with Av-ARR2PB-Bax by the TUNEL assay. In the absence of dihydrotestosterone, approximately 5.3% (95% CI 4.0% to 6.7%) of cells were TUNEL positive; in contrast, in the presence of dihydrotestosterone, approximately 55% (95% CI 51.8% to 60.0%) were positive, a statistically significant difference (P < .001). Dihydrotestosterone activated the mitochondrial pathway of apoptosis, shown by the cytosolic translocation of cytochrome c (Fig. 6, A, lane 3); activation of the caspase pathway, shown by cleavage of procaspase-3 (which produced the active fragment of 17 kDa) (Fig. 6, B, lane 5) and procaspase-7 (Fig. 6, B, lane 5); induction of DEVDase activity (data not shown); and by proteolytic digestion of the death substrate DFF (Fig. 6, B, lane 5). Apoptosis was activated only in dihydrotestosterone-treated cells. Cells infected with Av-ARR2PB-CAT did not activate apoptosis (Fig. 6, A, lanes 5–8; Fig. 6, B, lanes 2 and 3), whereas cells infected with the binary system activated the mitochondrial pathway of apoptosis by a dihydrotestosterone-independent mechanism (Fig. 6, A, lanes 9–12; Fig. 6, B, lanes 6 and 7).

For In Vivo Studies

Having demonstrated that Av-ARR2PB-Bax overexpressed Bax in a dihydrotestosterone-dependent way in every AR-positive prostate cancer cell line studied, we tested whether Av-ARR2PB-Bax could be used to induce apoptosis in an in vivo model of prostate cancer. Before treatment, the LNCaP tumors were 34.1 mm$^3$ (95% CI 25.1 mm$^3$ to 43.1 mm$^3$) (Fig. 7, A). Seven days after the last of four intratumoral Av-ARR2PB-Bax injections, the size of LNCaP tumors had decreased to 24.6 mm$^3$ (95% CI –2.5 mm$^3$ to 51.7 mm$^3$) (Fig. 7, A), a 30% decrease that was not statistically significant (P > .05) in view of the poor response in two of the six tumors (Fig. 7, B). By contrast, the size of the control tumors before treatment was 28.9 mm$^3$ (95% CI 12.7 mm$^3$ to 45.1 mm$^3$) (Fig. 7, A). Seven days after the last of four intratumoral Av-ARR2PB-Bax injections, the size of LNCaP tumors had decreased to 24.6 mm$^3$ (95% CI = –2.5 mm$^3$ to 51.7 mm$^3$) (Fig. 7, A), a 30% decrease that was not statistically significant (P = .5) in view of the poor response in two of the six tumors (Fig. 7, B). By contrast, the size of the control tumors before treatment was 28.9 mm$^3$ (95% CI = 12.7 mm$^3$ to 45.1 mm$^3$), and 7 days after the last of four intratumoral Av-ARR2PB-Bax injections, the size of LNCaP tumors had decreased to 24.6 mm$^3$ (95% CI = –2.5 mm$^3$ to 51.7 mm$^3$) (Fig. 7, A), a 30% decrease that was not statistically significant (P = .05).

The tumors treated with Av-ARR2PB-Bax were 8.5-fold smaller than the Av-ARR2PB-CAT-treated tumors (P = .002). There was an almost complete regression in four of six tumors that received intratumor injections of Av-ARR2PB-Bax (Fig. 7, B). Thus, this result suggested that treatment with ARR2PB-Bax not only reduced tumor size (comparison with the...
pretreatment size) but also prevented tumor growth (comparison between treatments).

Statistically significantly more TUNEL-positive cells were detected in tumors treated with Av-ARR2PB-Bax, average 23.3% (95% CI 21.1% to 25.6%) apoptotic cells, than in tumors treated with Av-ARR2PB-CAT, average 9.5% (95% CI 8.0% to 11.1%) apoptotic cells (P < .001). There was no macroscopic tissue damage evident in the area surrounding the tumor or in the liver, lungs, heart, or kidneys of mice in either group (data not shown).

**DISCUSSION**

We have used an adenovirus, Av-ARR2PB-Bax, with multiple regulatory features to drive prostate-specific, androgen-dependent, Bax-mediated apoptotic death in prostate cancer cell lines in vitro and in vivo. The prostate-specific probasin promoter targeted expression to prostate epithelium, whereas two ARRs within the promoter conferred AR sensitivity that was controlled by dihydrotestosterone. Thus, dihydrotestosterone treatment of AR-positive cells drove Bax expression that activated the mitochondrial apoptotic death pathway. Apoptosis occurred in AR-positive prostate cells that were androgen sensitive or androgen insensitive and was restricted to AR-positive prostatic epithelium, because AR-positive cell lines of extraprostatic origin and AR-negative cell lines of prostatic or extraprostatic origin were unaffected. Because this adenoviral construct also decreased the size of prostatic tumors in nude mice, it may be a useful therapeutic strategy for the treatment of prostate cancer in man.

We decided to overexpress Bax rather than a different proapoptotic protein for several reasons. First, Bax is a proven inducer of apoptosis when overexpressed in a variety of cell lines (6–10,34). Second, Bax inactivation is, itself, an important event in human carcinogenesis. For example, in colon and gastric cancers, Bax inactivation contributes to tumor progression by providing a survival advantage over cells containing the wild-type protein (35). In addition, Bax inactivation completely abolishes the induction of apoptosis by the chemopreventive drug sulindac in an experimental model of colon cancer (36). Ultimately, with continued treatment with sulindac, tumors that lack Bax expression are selected that have a survival advantage over wild-type clones because of the inability to engage the apoptotic machinery.

Our previous work (6) found that adenovirus-mediated Bax overexpression is lethal in all prostate cancer cell lines tested, independent of their AR status or androgen sensitivity (6). Other investigations (7–10) have shown that adenovirus-mediated Bax overexpression is lethal in many cell types. Thus, the development of a promoter specific for prostatic epithelium was mandatory to keep Bax from accumulating in extraprostatic tissue. Although the first-generation rat probasin promoter was specific
for prostate tissue, a previous study (11) found that it only weakly drives expression of linked cDNAs in vivo. However, the addition of a second copy of the ARR in the new ARR2PB promoter was sufficient to increase Bax overexpression by approximately 1300-fold, similar to that reported previously. Thus, the ARR2PB promoter has many of the targeting, regulatory, and potency properties required of a promoter for gene therapy for prostate cancer.

Several interesting observations relate to the requirement of the AR in androgen sensitivity. Stable transfection of the AR into AR-negative prostate cancer cells led to dihydrotestosterone-mediated Bax expression in PC-3 cells and, to a lesser degree, also in M12 cells. These results suggest that AR is only one of the many components related to androgen insensitivity in prostate cancer, and that when AR is reinserted, the degree of its ability to transcribe genes is cell type specific. In addition, because the ARR2PB promoter was unable to drive Bax expression and apoptosis in AR-positive cell lines of nonprostatic derivation, the mere presence of the AR was insufficient to confer dihydrotestosterone-inducible Bax expression after infection with the Av-ARR2PB-Bax construct. Lack of dihydrotestosterone-inducible Bax expression may simply reflect that, in AR-positive cell lines of nonprostatic derivation, the AR regulates the transcription of genes other than probasin, which is notoriously specific for prostatic epithelium.

In any event, taken together, these findings suggest that the differential ability of the AR to activate the ARR2PB promoter in various AR-positive cell lines may result from the disruption or uncoupling of com-

![Fig. 7. Effect of Av-ARR2PB-Bax on the size of LNCaP xenografts in vivo.](image-url)

**A)** In vivo experiments with the Av-ARR2PB-Bax. Mean volume ± 95% confidence interval of six subcutaneous LNCaP tumors xenografted in nu/nu uncastrated male mice and treated with four injections (1.9 × 10⁹ plaque-forming units/injection) of Av-ARR2PB-CAT or Av-ARR2PB-Bax, which were given at weekly intervals. Arrows indicate the times of administration of the virus. Asterisks denote statistical significance between the ARR2PB-Bax and the ARR2PB-CAT group at the same time point (P = .01, P = .0043, and P = .0024 at 2, 3, and 4 weeks of treatment, respectively, by two-sided paired Student’s t tests).

**B)** Image of the 12 tumors at the end of the treatment.
ponents of the AR signal transduction pathway, such as an accumulation of AR corepressors or a loss of AR coactivators.

Of interest, the ARR2PB promoter was effective in driving Bax expression in the AR-positive but androgen-independent cell line C4–2. This cell line represents a good experimental model of prostate cancer for patients who have failed to respond to androgen-ablation therapy (14). A fundamental question in androgen-independent cancers is whether the androgen-signaling pathway is still functional. Because androgen-independent cancers are unresponsive to further hormonal manipulations, it could be that abnormalities, such as those discussed above, have arisen in the portion of the AR-signaling pathway that specifically regulate apoptosis. Alternatively, AR-independent mitogenic pathways may have become dominant, eliminating the requirement for the AR. Our experiments indicate that the AR-signaling pathway is still functional in C4–2 cells and that their ability to grow in the androgen-depleted environment of castrated mice is possibly due to the selection of dominant-alternative mitogenic pathways or to ligand-independent activation of AR. That the ARR2PB promoter is inducible in an AR-dependent way in this cell line is promising for the future use of this promoter to drive gene expression in androgen-independent human prostate cancer because most primary and metastatic prostate cancers, including those that continue to grow following androgen ablation, do express the AR (38–42).

In vivo treatment with Av-ARR2PB-Bax was successful both in preventing tumor growth compared with control (Av-ARR2PB-CAT)-treated tumors and in promoting tumor regression compared with the pretreatment size. Tumors that received Av-ARR2PB-Bax had a statistically significant increase in the percentage of TUNEL-positive cells compared with tumors that received the control construct Av-ARR2PB-CAT. However, we performed the analysis only at one time point (i.e., in this study, 7 days after the last injection), which may have limited our ability to detect TUNEL-positive cells during the earlier most active time period of apoptotic death, which in the cell lines was at 24 hours. It is conceivable that, by the time of analysis, many cells may have already died by apoptosis and been removed from the tumor, which would contribute to the decreased tumor size. A statistically significant difference between the mean tumor volumes of the two groups was identified at weeks 2, 3, and 4 of the experiment (Fig. 7, A), and a complete regression of the tumor was observed in the majority of tumors (Fig. 7, B). Although the primary aim of this study was not to evaluate Av-ARR2PB-Bax toxicity, it is encouraging to note that no macroscopic damage was found in various organs and that mice survived 28 days of treatment without any apparent distress. A more comprehensive assessment of the toxicity associated with these constructs is planned, with histologic and immunologic evaluations in models of prostate cancer that use immunocompetent mice and with RNA analysis to detect human Bax expression in specimens obtained from various organs of the host animal.

It is likely that prolonged treatment or use of larger quantities of the virus will be more efficacious in the regression of prostate cancers. Thus, constructs, such as Av-ARR2PB-Bax, may, in the future, represent an additional weapon in the armamentarium to treat primary prostate cancers with gene therapy. The experiments shown in this study support a role for the use of genes that encode for proteins of the apoptotic pathway to induce thera-

deutic apoptosis and suggest that this approach is safe if specific promoters, such as ARR2PB, are used. These genes represent an evolution from previously used cytotoxic genes, such as herpes simplex virus–thymidine kinase (HSV-tk) (43), which required ganciclovir infusion for activity. Future experiments will evaluate if a bystander effect is present after intratumoral expression of Bax, as it is with HSV-tk. Nevertheless, the main challenge lying ahead is to develop technology to target death genes, such as Bax, to metastatic deposits of prostate cancer. This may eventually become possible with the availability of prostate-specific vectors.

References


(15) Plymate SR, Bae VL, Maddison L, Quinn LS, Ware JL. Reexpression of the type I insulin-like growth factor receptor inhibits the malignant phenotype of simian virus 40 T antigen immortalized human prostate epithelial cells. Endocrinology 1997;138:1728–35.


NOTES

Present address: F. Andriani, Department of Experimental Oncology, Molecular-Cytogenetics Unit, Istituto Nazionale dei Tumori, Milan, Italy. Present address: X. Li, Department of Medicine, McGill University and Royal Victoria Hospital, Montreal, PQ Canada. F. Andriani and B. Nan contributed equally to this paper.

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