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INTRODUCTION

To develop new therapeutic means to treat androgen-independent prostate cancer, we hypothesized that the apoptotic pathway of prostate cancer cells can be manipulated for therapeutic purposes. In support of our hypothesis, we presented preliminary data showing that a molecule of the apoptotic pathway, caspase 7, induced therapeutic apoptosis after adenoviral-mediated overexpression. This was observed *in vitro* in LNCaP and LNCaP-Bcl-2 cells, and *in vivo* in the prostate of normal mice, which were inoculated with a virus overexpressing caspase-7 under the control of the powerful viral promoter RSV (AvC7).

That adenoviral mediated caspase-7 overexpression has the ability to induce therapeutic apoptosis in these two cell lines has now been reported by our group in two papers published in Cancer Research (1, 2). Based on this preliminary data, in aim 1 we proposed to investigate whether adenovirus AvC7 has the ability to modify the natural history of prostate cancer in the TRAMP mouse model, which was developed in the laboratory of Dr. Greenberg at Baylor College of Medicine (3).

First generation adenoviral constructs have some potential shortcomings, for instance they are significantly immunogenic, and their ability to maintain expression of the therapeutic gene of interest may be limited to a maximum of 2-3 weeks before they are neutralized by the immune system of the host. In view of this, first generation adenoviruses can only be directly inoculated in a cancerous lesion, and cannot be used systemically to reach metastatic deposits. For this reasons we have proposed in aim 2 to construct a gutless helper-dependent (HD) second-generation adenovirus. As these constructs are completely devoid of proteins of viral origin, they are only minimally immunogenic (4), are not recognized by the immune system, and have the ability to produce the gene of interest up to > 2 years after their initial inoculation (5, 6). Our original argument was that such constructs would be more amenable to treat not only primary neoplastic formations, but also metastatic deposits, and that this could be achieved by possibly inoculating the animal with multiple injections given over a long period of time.

In the third specific aim of our application we proposed to study if an HD vector carrying the RSV-Caspase-7 cassette has the ability to shrink *sc* xenografts of prostate cancer cell lines, following direct inoculation in the tumor, or systemic injection through the tail vein.

BODY

Task 1

In task 1 of the original statement of work, we proposed to prove the concept that adenoviral-mediated overexpression of caspase-7 is associated with a change of the natural history of prostate cancer in TRAMP mice. We planned the following steps to conclusively demonstrate this point.

- 1) Identify the dose of virus infecting $\geq 90\%$ of prostatic epithelium of TRAMP mice using 10^8 to 10^{10} pfu of Av-GFP, each in three mice. (Technique: Identify and score GFP fluorescence in the prostate of these animals). (months 1-2)
- 2) Identify the dose of AvC7 causing maximal apoptosis of the prostate with minimal systemic toxicity. 18 TRAMP mice undergoing extensive analysis post-sacrifice (see table II A). (months 3-6)

- 1) Identify if multiple inoculations of the dose established in 2 are more effective than one single inoculation. Twelve TRAMP mice undergoing extensive analysis post-sacrifice. (see table IIB) (months 6-8)
- 2) Does treatment with AvC7 change the natural history of prostate cancer in TRAMP mice? 30 TRAMP animals treated with AvC7 or AvGFP according to the modalities established in 2 and 3 will undergo extensive analysis post-sacrifice (which will occur 15 weeks after initial inoculation as explained in table IIC). (months 8-15).

Step 1 of the investigation was initially performed using adenovirus Av-RSV-GFP, which consists of a green fluorescent protein cDNA driven by the RSV promoter. The experiment was done using the experimental conditions described in step 1. 24-week old TRAMP mice from our colony were used to perform these experiments. Following abdominal surgery, mice were injected with 10^8 to 10^{10} pfu of Av-GFP directly in the ventral prostate. 7 days post-surgery tissue was collected and paraffin-embedded. As we were unable to detect green fluorescence in this tissue by fluorescent microscopy or by immunohistochemistry, further experiments were done using adenovirus Av-RSV-caspase-3 (AvC3). The reason why we used the adenovirus overexpressing caspase-3 is because at the time of these experiments we had developed a very good caspase-3 immunostaining assay. Detection of caspase-3 in prostatic tissue was done using an



Fig 1: A. H&E staining of the prostatic adenocarcinoma in a 24-week old TRAMP mouse. B. Immunostaining for human caspase-3 in an adjacent section of the same tumor. Caspase-3 immunostaining is close to 100% when the virus Av-C3 is given at the concentration of 10^{10} pfu.

antibody for caspase-3 from Transduction Laboratories. With all concentrations tested there was a significant concentration of positive staining in the cells of these prostatic tumors (Fig. 1 panel B). Because at concentrations of virus between 10^8 to 10^{10} we did not detect major differences in the amount of positive immunostaining for caspase-3, we decided to use the concentration of 10^9 pfu for further studies. Step 1 of task 1 was completed in 6 months due to the technical difficulties in identifying GFP fluorescence in paraffin-embedded tissue.

Step 2 was done to identify the dose of AvC7 causing maximal apoptosis of the prostate with minimal systemic toxicity. These experiments were done using TRAMP mice at an early age, before they had developed prostate cancer as the animals described

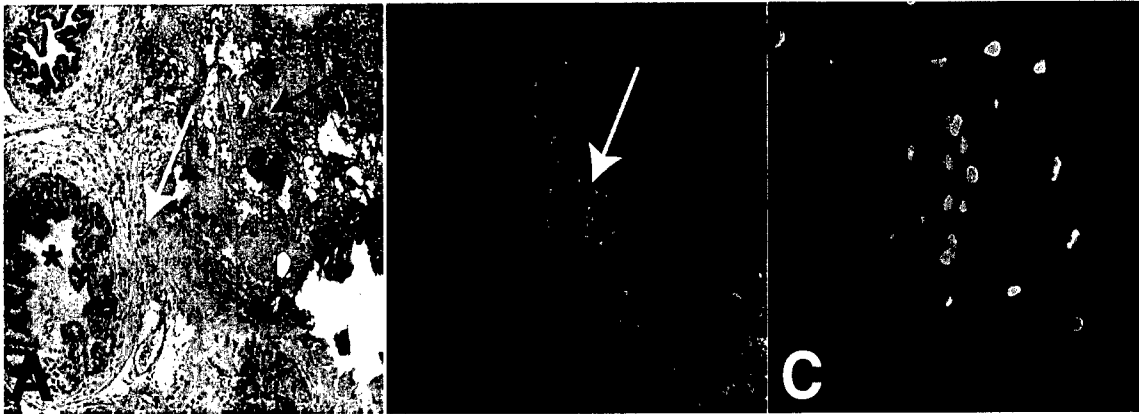


Fig. 2: Prostates were treated seven days earlier with one single inoculation of 10^9 pfu of AvC7. After seven days animals were sacrificed, the tissue was formalin fixed and stained with H&E, or by using TUNEL. A. H&E staining of a region showing normal glandular tissue (*) and tissue undergone dramatic morphological changes. B TUNEL of this region showing intense immunofluorescence in the area indicated both in A and B by the white arrow. C. Detail of the region indicated by the white arrow at higher magnification.

in Fig. 1. AvC7 was inoculated in the dorso-lateral prostate of these mice at concentration ranging from 10^8 to 10^{10} pfu's (each dose to 6 mice for a total of 18 animals).

Animals were sacrificed after 7 days. Analysis was done for: **a.** Wet weight of the prostates, which was $100 \text{ mg} \pm 20$, $120 \text{ mg} \pm 34$, $91 \text{ mg} \pm 10$ in the groups treated with 10^8 , 10^9 and 10^{10} pfu of virus (no statistical difference). **b.** Scoring of TUNEL + and - prostate cells to quantitate apoptosis. This was done by scoring for TUNEL positivity a total of 300 cells (50 cells in 6 microscopic fields) using a fluorescent microscope. TUNEL positive cells were $10\% \pm 8$, $17\% \pm 17$ and $20\% \pm 5$ in the groups treated with 10^8 , 10^9 and 10^{10} pfu of virus ($P = 0.01$ between the 10^8 and the 10^{10} group, while there was a non-significant difference between the 10^8 and 10^9 or 10^9 and 10^{10} groups). Figure

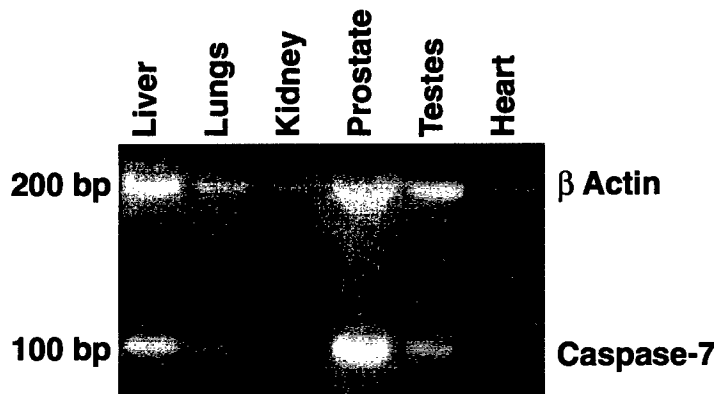


Fig. 3: PCR of genomic DNA extracted from various organs of mice injected intraprostatically with one inoculations of AvC7 (10^{10} pfu). A fragment of 200 bp of β -actin was amplified in the same reaction to control for the efficiency of the reaction. A human caspase-7 specific band was recovered from the prostate (the larger amount), testes and liver.

2 shows a prostate receiving 10^9 pfu of virus. The histologic structure of the prostate is dramatically altered by an injection with Av-C7 (panel A, black arrow), and significant degree of TUNEL positivity (panel B arrow) is present in the region between the area presenting normal histology (indicated by an asterisk) and the area with a histologic

obliteration of the prostatic structure (indicated by an arrow). In the H&E staining of panel A, the area of apoptosis is indicated by a white arrow. Note that the area undergoing histologic obliteration is not TUNEL

positive, possibly because by the time the tissue was harvested this area had already past

the phase of TUNEL positivity **c.** Macro and microscopic necroscopy to rule out systemic side effects [which were not identified (data not shown)], **d.** Genomic DNA was extracted from prostate, liver, lungs, kidneys, heart, and testes to rule out extraprostatic invasion of the virus by PCR, using one primer derived from viral sequences adjacent to the cloning site, and the antisense primer derived from sequences of the human caspase-7 cDNA (this should have amplified a band of 200 bp). Using concentrations of virus of 10^{10} pfu, we found that the large majority of the virus was detected in the prostate. However, significant amounts were also found in the liver (where adenoviruses tend to naturally accumulate) and in adjacent tissue such as the testes (Fig. 3). In contrast no extraprostatic diffusion of the virus was detected for concentrations of 10^8 or 10^9 pfu (not shown). These initial experiments have demonstrated that inoculation of 10^{10} pfu of AvC7 in normal prostates induces a significantly increased amount of apoptosis, but this was associated with accumulation of the virus in extraprostatic sites such as the liver and the testes. Thus, the concentration of 10^9 pfu's was chosen for Step 3. In step 3 we wanted to identify if multiple inoculations of the dose established in Step 2 were more effective than one single inoculation. For this part of the investigation we used twelve TRAMP mice. The first group of six was inoculated with one inoculation of 10^9 pfu of AvC7 in the prostate. According to our original proposal, the second group was initially supposed to receive four intraprostatic inoculations of the same dose of virus. Unfortunately this group of mice started to die after the third intraprostatic inoculation of the virus. This was most likely due to stress related to such a large number of surgeries, and consequently we were forced to start a second experiment in which we are planning to give two inoculations of virus instead of four. Due to this problem, Step 3 is not yet completed, and we think the results will be available by May-June 2001. As a result of these delays, Step 4 of this aim will be completed by the end of year 2 of the investigation.

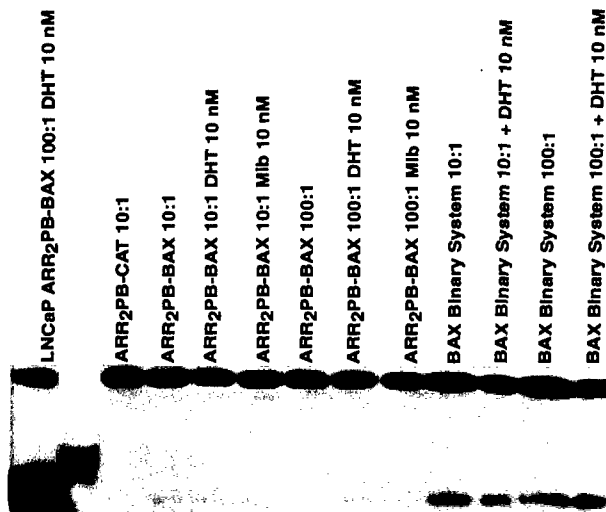


Fig. 4: PC-3 cells were infected at the indicated MOI with ARR₂PB-CAT (the control) or ARR₂PB-Bax, or the constitutively active binary system overexpressing Bax. Experiments were done in the presence or absence of DHT or of the synthetic androgen mibolerone. The first lane contains LNCaP cells infected with ARR₂PB-Bax at a MOI of 100:1 in the presence of DHT. The upper band represent β-actin (46 kDa), that was detected to ensure equal loading in each lane, the lower band (21 kDa) represents Bax. The experiment shows that ARR₂Pb does not drive Bax expression in this AR- cell line.

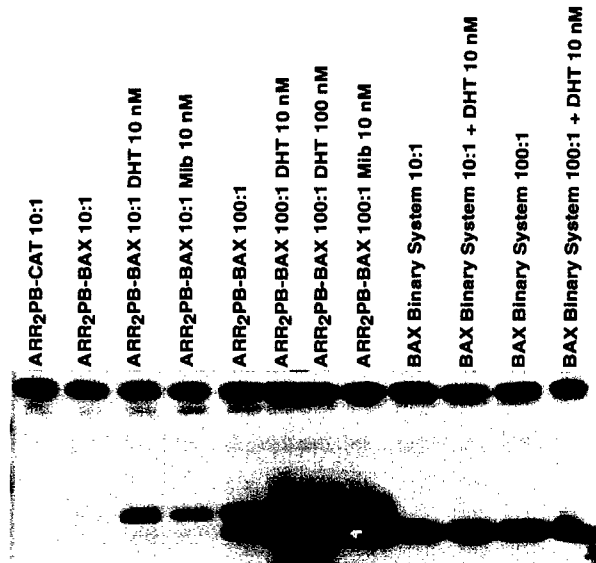


Fig. 5: LNCaP cells were infected at the indicated MOI with ARR₂PB-CAT (the control), ARR₂PB-Bax, or the constitutively active binary system overexpressing Bax. Experiments were done in the presence or absence of DHT or of the synthetic androgen mibolerone. The upper band represent β-actin (46 kDa), that was detected to ensure equal loading in each lane, the lower band (21 kDa) represents Bax. There is dramatic induction of Bax expression in a DHT/mibolerone-dependent way.

Task 2: To generate HD constructs containing C7 or GFP under the control of the 6 kb PSA promoter, and to validate the efficacy of the PSA promoter using PSA+ or - cell lines.

In task 2 of the original statement of work, we proposed to generate HD viruses carrying the cDNA for caspase-7 and GFP under the control of the 6 kb PSA promoter. Instead of the 6 kb PSA promoter, we have decided to use a third generation probasin promoter recently developed in the laboratory of Dr. Robert Matusik (7). Work of our laboratory now in revision at the Journal of the National Cancer Institute has demonstrated that this promoter induces the gene of interest (which is Bax in Fig. 4 and 5) in a dihydrotestosterone-dependent way only in AR+ cells of prostatic derivation by 1200-fold compared to control. Figure 4 shows that this construct is inactive in androgen receptor negative PC-3 cells, while figure 5 shows that this construct is dramatically inducible by DHT (or the non-metabolizable androgen mibolerone) in androgen receptor positive LNCaP cells. The work shown in Figures 4 and 5 is part of a concomitant project going on in the lab, which is mainly supported by the Veterans Administration. However, because the information generated in the context of this parallel investigation is so relevant for the decision to use the ARR₂PB instead of the 6 kb PSA promoter, we have inserted the submitted paper as an appendix in this progress report. A direct comparison between the ARR₂PB and the 6 kb PSA promoters was not done, but in view of the dramatic induction of the gene of interest that was obtained using ARR₂PB (Figure 5), we decided to use this promoter instead. Task 2 was mostly involved with the construction of second generation HD adenoviruses, which will be used in the experiments of aim 3 to induce therapeutic apoptosis of experimental prostate cancer after direct and systemic inoculation. At the present time we have done all the cloning

procedures and the homologous recombination in 293 cre4 cells. Viral particles have been just isolated, and we are on our way to screen these viruses for the presence of ARR₂PB-caspase-7, ARR₂PB-Bax and ARR₂PB-GFP sequences. The viruses will be subsequently used in cell lines to see if we can see an effect similar to that shown in Figure 4 and 5, and then will be used for the experiments discussed in aim 3 of the original application. Task 2 is in almost perfect timing with the original schedule that was proposed in the original application.

Task 3: To demonstrate that PSA-driven HD adenovirus express the genes of interest in a PSA-dependent way, and that PSA-dependent overexpression of caspase-7 causes apoptosis of PSA + cells after direct or systemic inoculation.

We have not yet started working on task. We do not anticipate to work on it until the end of this year, beginning of next year.

KEY ACCOMPLISHMENTS

- ◆ One of the key accomplishments of this initial year of the project is the demonstration that in normal prostatic epithelium caspase-7 overexpression has an apoptotic effect, and leads to histologic obliteration of the prostate after direct inoculation. It will be very important to test the efficacy of this vector in a sophisticated experimental model of prostate cancer such as the TRAMP model. This will be one of the key experiments of the incoming year.
- ◆ Another important observation is that high doses of the virus injected intraprostatically can be detected also in other organs, such as testes and liver. It should be pointed out that we could not detect any histologic abnormalities in the testes and liver of these animals. However, the important message from this experiment is that safety reasons may limit use of powerful apoptotic genes, unless one uses organ-specific promoters to express the gene of interest. We are addressing this important concern of safety by constructing HD viruses, which are less immunogenic, and the ARR₂PB promoter, which seems to be very powerful and specific for prostatic epithelium [(7) and Appendix I].

REPORTABLE OUTCOME

At this time the data described in this project report are not reportable. We need to complete specific aim 1 in its entirety before data can be reported as a manuscript.

- ◆ Data which are inserted in this project report have been presented in abstract form at the "Hormones and Cancer 2000" International Meeting in Port Douglas Australia (November 3-7, 2000), where the PI was an invited speaker (Abstract# 23: GENE THERAPY FOR THE INDUCTION OF THERAPEUTIC APOPTOSIS IN PROSTATE CANCER USING APOPTOTIC MOLECULES AND PROSTATE-SPECIFIC PROMOTERS. F Andriani, X-Y Le, J. Yu, B. Fang, S. Kasper, L. Denner, R. Matusik, M. Marcelli).
- ◆ Data have also been reported in abstract form at the AACR meeting in New Orleans Louisiana (March 24-28, 2001), where we had a poster presentation. (Abstract # 3706: GENE THERAPY FOR THE INDUCTION OF THERAPEUTIC APOPTOSIS IN PROSTATE CANCER USING APOPTOTIC MOLECULES AND PROSTATE-SPECIFIC PROMOTERS.

F Andriani, B. Nan, J. Yu, X-Y Le, B. Fang, S. Kagawa, N. Weigel, S. Kasper, R. Matusik, L. Denner, M. Marcelli).

- ◆ Data will also be presented at the International Meeting "Classical and Non-Classical Issues from Prevention to Treatment of Hormone-Related Tumors" which will be held at Erice, Italy from, May 1-6, 2001 where the PI is an invited speaker. (Abstract title; Controlling the apoptotic pathway in prostate cancer by B. Nan, J. Yu, F Andriani, Y. Zhang, S. Kasper, R. Matusik, L. Denner, M. Marcelli).

CONCLUSIONS

In conclusion, this initial year of the project has been a very fruitful one. We have initiated a number of experiments which should produce results by the end of the next year of funding. It is expected that we will understand whether the main hypotheses of this investigation are confirmed in the next 18 months of the investigation. Potential outcomes from this investigation are the development of new strategies to perform gene therapy, and of new prostate specific vectors.

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APPENDIX 1

This manuscript has been submitted to the Journal of the National Cancer Institute. We are sending this paper with this progress report because in aim 2 we have now decided to

use the ARR₂PB promoter as a system to direct gene expression to prostatic epithelium instead of the 6 kb PSA promoter. We have based this change on the data developed in this enclosed manuscript.

**THE THIRD GENERATION PROBASIN PROMOTER
ARR₂PB DRIVES BAX OVEREXPRESSION AND
APOPTOSIS IN ANDROGEN RECEPTOR POSITIVE
PROSTATE CANCER CELLS IN A
DIHYDROTESTOSTERONE-DEPENDENT WAY.**

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Key words: prostate cancer, apoptosis, probasin promoter, gene therapy.

Abbreviations: PB probasin, AR androgen receptor, PSA prostate-specific antigen, MMTV mouse mammary tumor virus, DHT dihydrotestosterone, TUNEL TdT-mediated-dUTP-X nick end labeling, DFF DNA fragmentation factor.

Abstract

Androgen-stimulated adenoviral overexpression of Bax induced apoptotic death in androgen receptor positive (AR+) prostatic epithelial cells. Hormone regulated, prostate targeted expression was achieved using the prostate-specific probasin (PB) promoter modified to contain additional androgen response elements (ARR₂PB), to drive adenoviral expression of Bax from the construct Av-ARR₂PB-Bax. No Bax expression occurred in AR+ cells of non-prostatic derivation, or in AR negative (AR-) cell lines of either prostatic or non-prostatic derivation. Stable transfection of an AR cDNA reconstituted AR signaling in PC-3 but not M12 cells, leading to androgen regulated Bax expression from Av-ARR₂Pb-Bax. Five injection of Av-ARR₂PB-Bax into LNCaP xenografts growing subcutaneously in intact mice caused a 30% regression compared to baseline, and a 89% reduction relative to continued tumor growth observed in xenografts injected with the control virus Av-ARR₂PB-CAT. These data show that the Av-ARR₂Pb-Bax construct induces therapeutic apoptosis *in-vitro* and *in-vivo* by activating the mitochondrial pathway of apoptosis in a variety of AR+ cells derived specifically from prostatic epithelium, thus conferring a safety margin by not affecting non-prostatic cells. This construct should be a significant addition to the *armamentarium* to treat prostate cancer.

Introduction

In previous studies, we have investigated the feasibility of manipulating molecules of the apoptotic pathway for therapeutic purposes by using experimental models of prostate cancer (1-5). We found that adenoviral-mediated Bax overexpression induced apoptosis within 24 hours post-infection in a large variety of prostate cancer cell lines, by activating the mitochondrial pathway of apoptosis (6). Furthermore, three injections of the Bax overexpression system into PC-3 cell tumors in nude mice *in-vivo* caused 25% regression in tumor size corresponding to a 90% reduction relative to continued tumor growth in animals injected with the control binary system expressing Lac-Z. Thus, adenoviral-mediated overexpression of Bax has proven in our and other investigations (7-10) to be an effective way to induce therapeutic apoptosis in experimental models of prostate or other cancers. Although powerful, this approach is not devoid of potential side effects as overexpression of a Bax cDNA driven by a constitutively active promoter could induce unwanted apoptosis in every site of accidental accumulation. Thus, development of a system driven by a promoter that is sufficiently powerful to overexpress the therapeutic gene in the target tissue, and sufficiently selective to be active only in prostatic epithelium, is mandatory when one wants to perform gene therapy using apoptotic molecules such as Bax. Previous works from other investigators have addressed the important problem of constructing adenoviral vectors to specifically overexpress the molecule of interest uniquely in the prostate. Although specific to prostatic epithelium, the first generation probasin (-426/+28 bp 5' upstream of the rat PB gene), PSA (650 bp 5' upstream of the human prostate specific antigen gene), MMTV (1.1 kb ClaI-truncated mouse mammary tumor virus long-term-repeat) promoters are generally less powerful than constitutively active viral promoter,

and their ability to induce a therapeutic effect in the target cell is uncertain (11). Other prostate specific reagents, including the osteocalcin promoter (12), and the prostate specific membrane antigen promoter and enhancer (13), have been identified but their efficacy *in-vivo* using adenoviral technology is at this time unknown. We decided to use the modified rat ARR₂PB promoter, which was developed in the Matusik laboratory (14) by subcloning one copy of the PB sequence -244 to -96 bp (Androgen Response Region ARR) upstream of the endogenous -286/+28 PB promoter. This composite promoter now contains two ARRs. Transfections of reporter genes driven by ARR₂PB were followed by > 200-fold induction of reporter gene activity in an androgen-dependent manner in AR+ cell lines derived from prostatic epithelium (14). Additionally, the same promoter remained highly specific for prostatic epithelium in transgenic mice (14). In the current study, we have hypothesized that an adenoviral particle containing the ARR₂PB promoter linked to the Bax cDNA may have a number of advantages over previous constructs developed for gene therapy of prostate cancer. First, unlike other constitutively active promoters, ARR₂PB should be specific to prostatic epithelium. Second, unlike previous prostate-specific promoters, ARR₂PB should be powerful enough to drive adequate overexpression of the therapeutic gene to elicit a prompt therapeutic effect. Third, overexpression should be DHT (dihydrotestosterone)-inducible.

Using a large number of AR+ or AR- cell lines from a variety of tissues, we performed experiments to establish: 1. if an adenovirus containing the cDNA of Bax driven by ARR₂PB can overexpress the gene of interest uniquely in AR+ prostatic epithelium, 2. if this process is DHT-dependent, 3. if this occurs also in AR+ but

androgen-independent cell lines, and 4. if overexpression of the therapeutic gene is followed by apoptosis *in vitro* and *in vivo*.

MATERIAL AND METHODS

Materials

Fetal bovine serum and tissue culture media were from Life Technologies, Inc. (Frederick, MD). Chemicals were from Sigma (St.Louis, MO) unless stated otherwise. Antibodies and other reagents for apoptosis assays have been described previously (1-5). The full length Bax cDNA containing a HA tag at the NH₂ terminus was a gift of Dr. Korsmeyer (Harvard University Medical School). The antibody to detect cleaved caspase-3 was from Cell Signaling Technology (Beverly, MA)

Cells Lines

Several cell lines from different organs were utilized. Prostate cancer derived LNCaP, PC3, DU-145, TsuPr(1) have already been described (3). Prostate cancer-derived C4-2 (15) (maintained in 80% DMEM low glucose and 20% Kaighn's F12 with 5% of heat inactivated FBS supplemented with Insulin [5µg/ml, Sigma], T3 [13.65 pg/ml, Sigma], apotranferrin [5 µg/ml, Sigma], d-Biotin [0.244 µg/ml, Sigma], adenine [25µg/ml, Sigma] and 1% P&S), were purchased from UroCor Inc. (Ok). Prostatic epithelium-derived M12 cells (16) (maintained in RPMI 1640, 5% FBS and 1% P&S) were a gift of Dr. Steven Plymate (University of Washington Medical School, Seattle WA). PC-3-AR and M12-AR were clones of PC-3 and M12 cells stably transfected with an androgen receptor cDNA¹. Breast cancer derived MDA-MB-453 (17), MDA-MB-231 (17) and ZR-75 (18) (all maintained in IMEM (Zn²⁺ option) with 10% FBS, 1% P&S, and 1% of glutamine) were a gift of Dr. Powell Brown (Baylor College of Medicine). Melanoma derived BII (19) were a gift of Dr. Estela Medrano (Baylor College of

¹ Marcelli et. al.: unpublished

Medicine). Pancreatic cancer derived Panc-I (20) (maintained in DMEM + 10% FBS and 1% P&S) were from the ATCC. Genital skin fibroblasts strain 881 (21), 1003 (22) and 1017 (22) (all maintained in MEM + 10% FBS and 1% P&S) have been previously described. Low passage 293 cells (maintained in IMEM zinc option with 10% HI-FBS, 2mM L-glutamine and 1% P&S) were purchased from Microbix Biosystem (Toronto, Canada).

Preparation of Av-ARR₂PB-Bax

Adenoviral particles containing the human Bax cDNA linked to the ARR₂PB promoter were prepared according to the technology previously reported (1). Briefly, a full-length HA-tagged BAX cDNA was subcloned downstream of the ARR₂PB promoter in the background of the Bluescript plasmid (KS+) (Stratagene, San Diego CA). The ARR₂PB-Bax cassette was then cut with the restriction endonucleases Xba-I (blunted) and Xho-I and subcloned in the shuttle plasmid pXCJL-1 poly A+ cut with EcoR-V and Xho-I to obtain construct pXCJL-1-ARR₂PB-Bax-poly-A. pXCJL-1-poly-A (a gift of Dr. BaBie Teng, UT Medical School, Houston TX) has a PBR322 backbone and contains the human adenovirus type 5 (Ad5) 59-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 Ad5 sequence from nucleotide positions 3328 to 6246 serving as homologous recombination fragment. The recombinant adenovirus Av-ARR₂PB-Bax was prepared by cotransfecting the pXCJL-1-ARR₂PB-Bax-poly-A with pJM17 (23), which contains a full-length adenoviral genome, into low passage 293 cells (24) seeded onto a 60-mm culture dish the day before transfection at a density of 2×10^6 cells/dish by the calcium phosphate coprecipitation

method. Two weeks after transfection, recombinant adenoviral plaques were picked, propagated, and screened for ARR₂PB-Bax sequences by polymerase chain reaction. Adenoviral particles (named Av-ARR₂PB-Bax) that contained ARR₂PB-Bax were purified by a large-scale purification method described elsewhere (2).

Other Adenoviral constructs

Adenovirus Av-ARR₂PBCAT², containing the CAT reporter gene under the control of ARR₂PB, was used as a control for Av-ARR₂PB-Bax. The binary system for the overexpression of Bax consists of two adenoviruses (Ad/PGK/GV16 and Ad/GT-Bax) (9). The first (Ad/PGK/GV16) produces a powerful transcription factor, the GAL4-VP16 fusion protein under the control of the constitutively active PGK promoter. The second (Ad/GT-Bax) produces Bax under the control of a GAL/TATA minipromoter. Thus, the constitutively produced GAL4-VP16 binds the GAL/TATA minipromoter and drives transcription of Bax.

Experimental protocol

Two days before infection 1×10^5 cells were seeded in a six well plate. On the day of the infection, one of the six wells was trypsinized and the cells counted. This information was used to infect each cell line at the desired multiplicity of infections (MOI). Infections were carried out in a 5% CO₂ incubators at 37° for 1 hour using 500 µl of infection medium (the same medium used for each cell line + 2% FBS and 1% P&S) on a rocker. Pilot experiments with a virus expressing the GFP (green fluorescent protein) cDNA (Av-GFP) under the CMV promoter determined that the optimal MOI for most cell lines was 100:1 (data not shown). However to obtain a 100% positive GFP

² Kasper et. al.: unpublished

expression, some cells (such as the genital skin fibroblasts, MDA-MB-453 and MDA-MB-231 cells) had to be infected at MOI's of 500:1, while others such as the PC-3 required a MOI of 1000:1. Infection with the binary system were done using a ratio of 2:1 of Ad/GT-Bax vs. Ad/PGK/GV16 at a final MOI of 100:1 (or higher when requested). Each experiment was performed in regular FBS (or in charcoal stripped FBS when indicated so). One hour post-infection, cells were treated with or without dihydrotestosterone (DHT) at the concentration of 2 nM. In some experiments, increasing concentrations of DHT (ranging from 0.01 nM to 100 nM) were used in LNCaP cells growing in regular or charcoal stripped serum. In some experiments the non-metabolizable androgen mibolerone was used at the concentration of 2 nM.

Analysis of the apoptotic pathway

Two days post-infection with Av-ARR₂PB-Bax (in the presence or absence of 2nM DHT), or Av-ARR₂PB-CAT (in the presence or absence of 2 nM DHT), and 24 hours post infection with the binary system overexpressing Bax (in the presence or absence of 2nM DHT), cells were harvested and analyzed for Bax overexpression, procaspase-7 and cleaved caspase-3 expression, cytochrome c subcellular localization, DFF cleavage, DEVDase activity and TUNEL, using the techniques previously described (1-4).

In vivo studies

5 x 10⁵ LNCaP cells dispersed in RPMI 1640 and 20% Matrigel were xenografted subcutaneously in intact nu/nu mice (2 tumors in each mice, for a total of 6 tumors). Thirty days later, when tumors averaged a size of 30 mm³, treatment was started using weekly injection of 1.9 x 10⁹ pfu of virus (a total of 5 injections in five weeks). Six

tumors were injected with Av-ARR₂PB-CAT (the control group), and six with ARR₂PB-Bax (the treated group). Tumors were measured weekly using a caliper, and the volume calculated with the equation $m_1^2 \times m_2 \times 0.5236$ (where m_1 and m_2 are the smallest and largest diameter) (25). At the end of the treatment, animals were sacrificed, the tumor excised and weighted, and an autopsy was performed. Statistical analysis was done using two-tailed paired and unpaired Student's t tests.

RESULTS

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

ARR₂PB-Bax infection in prostate cancer and AR+ LNCaP cells

Infection of LNCaP cells with ARR₂PB-Bax at a MOI of 100:1 and stimulation with increasing concentrations of DHT (0.01 to 100 nM) produced a dose-dependent induction of Bax expression (Fig. 1B). The degree of Bax expression and induction was essentially similar in the presence of normal or charcoal-stripped serum (compare Fig. 1B and C). However, peak expression of Bax was obtained at lower concentrations of DHT (0.1 vs. 1 nM) in cells treated with charcoal stripped serum (Fig. 1B and C).

While at a MOI of 10:1 Bax expression was detectable only after addition of DHT, at a MOI of 100:1 Bax was expressed also in the baseline state, and further amplification was noticed after addition of DHT or Mobilerone (Figure 2A). Densitometric analysis of this experiment (normalizing Bax to β actin) demonstrated that at a MOI of 100:1 + DHT, Bax was expressed approximately 1300-fold more compared to baseline (Fig. 2A, compare lane 10 and 5). Furthermore, Bax was expressed 7-fold more compared to cells infected with a MOI of 100:1 of the binary system overexpressing Bax in a constitutive way (Figure 2A compare lane 10 and 14).

The two bands migrating as a doublet in Av-ARR₂PB-Bax-infected cells represent a 23 kDa form of Bax containing the HA tag at his NH₂-terminus, and the 21 kDa wild

type form (Fig. 2B lane 1). We base this conclusion on the experiment shown in Fig 2B, in which an anti-HA antibody could recognize only the higher band overexpressed in Av-ARR₂PB-Bax-infected cells (lane 3), and no bands in cells infected with the binary system (lane 4). In contrast, an anti-Bax antibody (Figure 2B) could recognize both the upper and lower form of Bax overexpressed by Av-ARR₂PB-Bax (lane 1) and the only band overexpressed by the binary system (lane 2). Thus, two forms of the Bax protein are synthesized in Av-ARR₂PB-Bax-infected cells. One form is initiated from the first methionine of the construct and incorporates the HA tag. The second form is initiated from the first methionine of the Bax cDNA, and the resulting band comigrates with the band produced in cells infected with the binary system, which does not have a HA tag in the Bax cDNA.

ARR₂PB infection in AR- prostate cancer cells PC-3, DU-145, TsuPr(1) and M12

Bax expression was not inducible in AR- cell lines of prostatic derivation such as PC-3 (Fig. 2C), DU-145, TSU-Pr(1) and M12 (data not shown) following infection with Av-ARR₂PB-Bax (MOI 100:1 except PC-3 where a MOI of 1000:1 was used) before or after addition of DHT. In contrast, in each cell line Bax overexpression could be achieved upon infection with the constitutively active binary system. PC-3 and M12 cells were then stably transfected with an AR expression vector, and selection was performed in G418-containing medium. The resulting AR+ cell lines PC-3-AR and M12-AR (Fig. 1) were infected with Av-ARR₂PB-Bax. Although not to the same extent as in LNCaP cells, Bax expression could be induced by DHT after infection with Av-ARR₂PB-Bax in PC-3-AR cells (Fig. 3A). In contrast, induction of Bax was not detected in M12-AR cells

(Fig. 3B). Infection with the binary system was associated with Bax overexpression in both PC-3-AR and M12-AR regardless of DHT addition (Fig. 3A and B).

Av-ARR₂PB-Bax infection in AR+ androgen-independent cell lines

To determine if Bax is overexpressed in cells that proliferate in the absence of androgens, we used C4-2 cells. These LNCaP-derived cells unlike their native counterpart 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+ androgen independence (15). Bax expression was dramatically induced in C4-2 cells following infection with ARR₂PB-Bax and treatment with DHT, confirming that these cells have maintained an intact AR signaling pathway. The degree of induction was essentially similar to what was observed in control cells treated with a similar MOI of the binary system (Fig. 3C). No induction of Bax was seen in control cells infected with Av-ARR₂PB-CAT (not shown).

Infection of Av-ARR₂PB-Bax in AR+ cell lines of non-prostatic derivation

Experiments were performed in cell lines derived from other tissues to verify if the promoter ARR₂PB is specific to AR+ prostatic epithelium. AR+ [MDA-MB-453 and ZR-75-1 (not shown)] or AR- (MDA-MB-231) cell lines derived from breast cancer were utilized (Fig. 4A, B), in addition to three AR+ genital skin fibroblasts cell lines. One of these (1017, Fig. 4C) was from a normal individual, one from a patient affected by complete androgen insensitivity due to a mutation of the DNA-binding domain associated with transcriptional silencing of AR (881, Fig. 4D) (21), and one (strain 10003, data not shown) from a patient affected by a Reifenshtein phenotype, containing a hormone-binding domain mutation causing a qualitative defect of DHT binding (22). Infection

with Av-ARR₂PB-Bax and treatment with DHT was not followed by induction of Bax expression in any of these cell lines (Fig 4). This applied to both AR+ and - cell lines, regardless of whether AR was wild type or mutated. In contrast the constitutively active binary system elicited a significant degree of Bax overexpression in every cell line.

Infection of Av-ARR₂PB-Bax in cell lines derived from other organs

Other AR- cell lines derived from other tissues, such as melanoma (IIB, data not shown) and pancreatic cancer (Panc-1, data not shown) were used to rule out the possibility that ARR₂PB drives gene transcription in cells of non-prostatic derivation. Again, while no overexpression of Bax could be detected in cells infected with Av-ARR₂PB, significant degree of Bax overexpression was detected in cells infected with the binary system.

Induction of apoptosis in LNCaP cells

We next investigated if ARR₂PB-induced Bax overexpression was associated with apoptosis in LNCaP cells. Approximately 55% of LNCaP cells underwent programmed cell death (as measured by TUNEL staining [data not shown]) 48 hours post-infection with Av-ARR₂PB-Bax in a DHT-dependent way. This was achieved through a DHT-induced activation of the mitochondrial pathway of apoptosis, as shown by the cytosolic translocation of cytochrome c (Fig. 5A lane 3), followed by activation of the caspase pathway, shown by cleavage of procaspase-3 [which produced the active fragment of 17 kDa (Fig 5B lane 5)] and -7 (Fig. 5C lane 5), an eight-fold induction of DEVDase activity (data not shown), and by proteolytic digestion of the death substrate DFF (Fig. 5D lane 5) only in DHT-treated cells. Cells treated with the control virus Av-ARR₂PB-CAT did not activate this pathway (Fig. 5A lanes 5-8, Fig. 5B, C and D, lanes 2-3), while cells treated

with the binary system underwent apoptosis by a DHT-independent mechanism (Fig. 5A lanes 9-12, 5B, C and D, lanes 6-7).

In-vivo studies

Having demonstrated that the binary system overexpressed Bax and induced apoptosis in every AR+ prostate cancer cell line studied, we tested this system in an *in-vivo* model of prostate cancer. As shown in Fig. 6A, five intratumoral injection with Av-ARR₂PB-Bax caused a 30% regression of LNCaP tumors that was evident after 35 days of exposure to the Bax virus and 7 days after the last injection. This represented an 8.5-fold reduction in tumor volume compared to those treated with the control virus Av-ARR₂PB-CAT ($p < 0.0006$). There was an almost complete disappearance of the lesion in 4 of the 6 tumors (Fig, 6B). Overall, tumor size decreased by 30% compared to pretreatment in the group treated with Av-ARR₂PB-Bax. No macroscopic damage was evident in the area surrounding the *sc* tumor, the liver, lungs, heart and kidneys of the animals in both groups (data not shown).

DISCUSSION

We have used an adenovirus, ARR₂PB, 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 specifically targeted expression to prostate epithelium, while the addition of a second AR-responsive region within this promoter conferred further AR sensitivity that was controlled by DHT. Thus, DHT treatment of AR+ cells drove Bax expression that led to engagement of the mitochondrial apoptotic death pathway. This occurred in AR+ prostate cells that were either androgen-sensitive or -insensitive, and was restricted to AR+ prostatic epithelium since AR+ cell lines of extraprostatic origin, as well as AR- lines of prostatic or extraprostatic origin, were unaffected. Since the use of this virus also decreased prostate cell tumor size in nude mice, this may be a useful therapeutic strategy in prostate cancer in man.

We decided to overexpress Bax among many other pro-apoptotic genes for at least two reasons. First, this molecule is a proven inducer of apoptosis upon overexpression in a variety of cell lines (6-10,26). Second, Bax inactivation is itself an important event in human carcinogenesis. For instance, in colon and gastric cancer, Bax inactivation contributes to tumor progression by providing a survival advantage over cells containing the wild type gene (27). In addition, inactivation of Bax completely abolished induction of apoptosis by the chemopreventive drug sulindac in an experimental model of colon cancer (28). Ultimately, Bax^{-/-} clones are selected for by this chemopreventive regimen and have a survival advantage over wild type clones due to the inability to engage the apoptotic machinery.

Our previous work demonstrated that adenoviral-mediated Bax overexpression is lethal in all prostate cancer cell lines tested independent of AR status or androgen sensitivity. However, other investigations have shown that adenoviral-mediated Bax overexpression is lethal in many cell types (7-10). Thus, development of a promoter specific for prostatic epithelium was mandatory to keep this lethal molecule from

accumulating in extraprostatic tissue. While the first generation rat probasin promoter is specific for the prostate, previous studies have shown it only weakly drives expression of linked cDNAs in vivo (11). However, two copies of the androgen responsive sequence in the new ARR₂PB promoter were sufficient to increase Bax overexpression by approximately 1300 fold in agreement with a previous report (14). Thus, ARR₂PB exhibits many of the targeting, regulation, and potency properties required of a promoter for gene therapy of prostate cancer.

Several surprising observations relate to the requirement of the AR in androgen sensitivity. Stable transfection of the AR into AR- cells caused DHT-mediated Bax overexpression in PC-3 cells but not M12 cells. These results suggest that loss of the AR is only one of the many components of androgen insensitivity in prostate cancer. In addition, since the ARR₂PB was unable to drive Bax transcription and apoptosis in AR+ cell lines of non-prostatic derivation, the mere presence of the AR is insufficient to confer DHT-induced apoptosis following infection with the construct Av-ARR₂PB-Bax. This may simply reflect that in these cells AR regulates transcription of genes other than probasin, which is notoriously specific for prostatic epithelium (29). In any event, these findings suggest that the inability of either heterologous expression of the AR in AR- cells of prostatic derivation, or the presence of the AR in non-prostatic cells to transcribe genes driven by the ARR₂PB promoter may result from disruption or uncoupling of components of the AR signal transduction pathway. These may include accumulation of AR co-repressors, or loss of regulators of the transcriptional machinery that positively affect ARR₂PB activity.

Interestingly, ARR₂PB was effective in driving Bax expression in the AR+ but androgen independent cell line C4-2. This cell line represents a good experimental model of prostate cancer for patients who have failed androgen ablative treatments (15). A fundamental question in androgen-independent cancers is whether the androgen signaling pathway is still functional. Because these patients are unresponsive to further hormonal manipulations, it could be that abnormalities such as those discussed above have arisen in the AR signaling pathway that specifically regulate apoptosis. Alternatively, AR-independent mitogenic pathways may have become dominant so that the AR is redundant. Our experiments indicate that the AR signaling pathway is still active in C4-2 cells, and that their ability to grow in the androgen depleted environment of castrated mice is possibly due to selection of dominant alternative mitogenic pathways. The fact that ARR₂PB is induced in an AR-dependent way in this cell line is promising for future use of this promoter to drive gene expression in androgen-independent human prostate cancer, as most primary and metastatic prostate cancers, including those that continue to grow following androgen ablation, do express the androgen receptor (30-34).

In-vivo treatment with Av-ARR₂PB-Bax was successful both in preventing tumor growth, and in promoting tumor regression in the LNCaP xenograft model. In the majority of instances, a complete regression of the tumor was observed. It is likely that more prolonged treatment or use of larger quantities of the virus will bring about a more complete eradication of these tumors. 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 of the apoptotic pathway for the induction of therapeutic apoptosis, and

suggest the safety of this approach if specific promoters such as ARR₂PB are used.

These genes represent an evolution from previously used cytotoxic genes such as HSV-tk, which requires Ganciclovir infusion for activity. Future experiments will evaluate if a significant bystander effect is present following intratumoral transduction of Bax, as it is when HSV-tk is used. 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 a possibility when prostate-specific vectors become available.

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LEGENDS

Figure 1: A. Immunoblot showing the AR status of the cell lines used in the study. Standard curve of Bax overexpression obtained in LNCaP cells growing in normal (B) or charcoal-stripped (C) Fetal Bovine Serum. Cells were infected at a MOI of 100:1 and stimulated with the indicated concentrations of DHT for 24 hours. Cell lysates were then subjected to Western analysis using antibodies for Bax and β -actin.

Fig. 2: Immunoblot analysis of LNCaP (A) and PC-3 (C) cells following infection with Av-ARR₂PB-CAT (lanes 1-4), Av-ARR₂PB-Bax (lanes 6-11) and with the constitutively active binary system (Ad/PGK/GV16 and Ad/GT-Bax) (lanes 12-15) compared to control uninfected cells (lane 5). LNCaP cells were infected at MOI's of 10:1 (lanes 1-2, 6-8 and 12-13) or 100:1 (lanes 3-4, 9-11, and 14-15) while PC-3 were infected at MOI's of 100:1 (lanes 1-2, 6-8 and 12-13) or 1000:1 (lanes 3-4, 9-11, and 14-15). Cells were then stimulated in the absence (lanes 1, 3, 5, 6, 9, 12, 14) or presence of saturating concentration of DHT (lanes 2, 4, 7, 10, 13, 15) or mibolerone (lanes 8 and 11). After 24 hours cell lysates were subjected to Western analysis with antibodies for Bax and β -actin. B: 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 DHT. Cells were then harvested, and lysates were subjected to Western analysis using an anti-Bax (lanes 1-2) or anti-HA antibody. While the anti-Bax antibody recognizes both the 23 and 21 kDa bands, the anti-HA antibody only recognizes the larger 23 kDa band. Each experiment was performed a minimum of three times.

Fig. 3: Immunoblot analysis of PC-3-AR (A), M12-AR (B) or C4-2 (C) cells following infection with Av-ARR₂PB-Bax (lanes 2-3) and with the constitutively active binary system (Ad/PGK/GV16 and Ad/GT-Bax) (lanes 4-5) compared to control uninfected cells (lane 1). Cells were infected at MOI's of 100:1 (except PC-3 AR which were infected at a MOI of 1000:1) and stimulated in the absence (lanes 1, 2, 4) or presence of saturating concentration of DHT (lanes 3, 5). After 24 hours cell lysates were subjected to Western analysis with antibodies for Bax and β -actin.

Fig. 4: Immunoblot analysis of MDA-MB-453 (A), MDA-MB-231 (B), 1017 (C) and 881 (D) cells following infection with Av-ARR₂PB-Bax (lanes 2-3) and with the constitutively active binary system (Ad/PGK/GV16 and Ad/GT-Bax) (lanes 4-5) compared to control uninfected cells (lane 1). Cells were infected at MOI's of 500:1 and stimulated in the presence or absence (lanes 1, 2, 4) of saturating concentration of DHT (lanes 3, 5). After 24 hours cell lysates were subjected to Western analysis with antibodies for Bax and β -actin.

Fig 5: Dissection of the mitochondrial apoptotic pathway in LNCaP cells infected with Av-ARR₂PB-CAT, Av-ARR₂PB-Bax and with the constitutively active binary system (Ad/PGK/GV16 and Ad/GT-Bax) and treated with or without DHT compared to control uninfected cells. A: 48 hours post-infection cell subfractionation was done, and the mitochondrial and cytosolic fractions were subjected to Western analysis with antibodies for cytochrome c and β -actin. B, C and D: Whole cell lysates were subjected

to Western analysis using antibodies for active and inactive caspase-3, procaspase-7 and DFF, respectively, plus β -actin. Note that the antibody for caspase-3 recognizes procaspase-3 and caspase-3 subfragments generating during apoptosis (these are indicated by the asterisks, and the two smaller bands correspond to a 19 kDa form consisting of the prodomain + the large active subunit, and to a 17 kDa form corresponding to the large active subunit). In contrast, the antibody for caspase-7 only recognizes the proform of caspase-7, which disappears following cleavage during apoptosis.

Fig. 6: A: Mean $\pm$ SD of the volume of six *sc.* LNCaP tumors xenografted in nu/nu mice and treated with five injection of Av-ARR₂PB-CAT or Av-ARR₂PB-Bax given at weekly intervals. B: image of the 12 tumors at the end of the treatment. There was statistically significant difference ($p < 0.0006$) in the size of tumors treated with Av-ARR₂PB-Bax vs. Av-ARR₂PB-CAT.

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