Adenovirus-mediated Tissue-targeted Expression of a Caspase-9-based Artificial Death Switch for the Treatment of Prostate Cancer

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ABSTRACT

Clinical experience with suicide gene therapy for prostate cancer using first-generation approaches has provided a basis for developing improved strategies. Given the low proliferation rate exhibited by prostate cancer, one improvement would be to develop suicide genes that effectively kill both dividing and nondividing cells. A second improvement would be to restrict cytotoxicity to prostate cancer cells, limiting injury of nondiseased tissue. Here we describe a novel approach to achieving both goals based on: (a) the use of a small, but potent, prostate-specific composite promoter, ARR:PB, based on the rat probasin gene; and (b) the use of a powerful artificial death switch, called inducible caspase-9 (iCaspase-9). ARR:PB includes two copies of the androgen response region (ARR), each containing two androgen receptor (AR)-binding sites, placed upstream of the probasin promoter elements necessary for basal transcription. Because iCaspase-9 contains two binding sites for the dimeric ligand, AP20187, administration of chemical inducers of dimerization leads to aggregation and caspase activation, followed by rapid apoptosis in both dividing and nondividing cells. Using both reagents, we constructed two novel adenoviruses (ADVs), ADV:ARR:PB-iCasp9 expressing iCaspase-9 and control ADV:ARR:PB-EGFP expressing enhanced green fluorescent protein (EGFP). We demonstrate that tissue-specificity is not sacrificed in an ADV backbone because the marker protein, EGFP, is expressed in R1881-stimulated ADV:ARR:PB-EGFP-transduced LNCaP cells but not in AR−PC-3, 293, HuH-7, U-87, and MCF-7 cells. Similarly, Pro:iCaspase-9 is expressed in ADV:ARR:PB-iCasp9-infected LNCaP cells after R1881 administration and is activated after AP20187 administration.

In vitro experiments revealed rapid and efficient iCaspase-9-induced apoptosis of LNCaP cells in both an R1881- and AP20187-dependent manner. Only 28, 8, and 0.5% survival of LNCaP cells was seen at multiplicities of infection of 2, 10, and 25, respectively. Furthermore, at a multiplicity of infection of 10, extraordinary sensitivity to AP20187 was seen (IC50 of 3 pm). In vivo experiments showed that ADV:ARR:PB-iCasp9 induced apoptosis in LNCaP but not in HuH-7 xenograft tumors in an AP20187-dependent manner. Furthermore, a simple i.p. injection of AP20187 dramatically suppressed LNCaP tumor growth in nude mice and led to a significantly increased host survival. This study demonstrates the feasibility of using tissue-specific expression of cell cycle-independent iCaspases as a nonmutagenic alternative modality for prostate cancer suicide gene therapy.

INTRODUCTION

Gene therapy for prostate cancer has moved rapidly into a developmental phase in which practical considerations for safety and efficacy have dominated efforts. Because of their wide range of infectivity, high achievable titers, and relatively low risk for secondary mutagenesis, recombinant ADV1 has been the vector of choice for studies involving intratumoral injection. However, the death in 1999 of a gene therapy patient after intravascular ADV delivery has tempered the ADV euphoria and refocused efforts to engineer appropriate safety mechanisms into ADV-mediated gene therapy strategies (1). These efforts have encompassed varying approaches to restrict viral tropism and transgene expression to target tissue. For example, by modifying the coxsackie/ADV receptor ligand on the ADV envelope, several groups have attempted to redirect the bulk of systemically released virus from the liver to specific target tissues (2, 3). Others have placed the E1 region, required for viral replication, under the transcriptional control of tissue-specific promoters, giving rise to tissue-restricted cytolytic viruses (4). Alternatively, the E1B/p55 region, necessary for viral replication in cells with intact p53 signaling, has been deleted, leading to a cytolytic virus with tumoricidal tendencies (5). Lastly, in replication-defective ADV, the entire E1 region, and often other sequences (e.g., E3 and E4 regions), has been completely replaced with cytotoxic genes. For the treatment of prostate cancer, these ADVs have included tumor suppressor genes (6–8), “suicide” genes (9, 10, 11), immunomodulatory genes (12, 13), or other therapeutic genes. By placing these transgenes under the transcriptional control of tissue-specific promoters, one should be able to improve safety and avoid autoimmunity or bystander toxicity.

Although a number of intact promoters from prostate-specific genes have proven to maintain prostate cell specificity, the level of gene expression that can be achieved is typically much less than the ubiquitously active reference CMV promoter/enhancer from CMV limiting their clinical utility (14–16). Therefore, several composite prostate-specific promoters/enhancers based on RPB, PSA, and glandular hK2, comprised of multiple androgen-responsive enhancer elements upstream of the prostate-specific minimal promoters, have been developed by others and us (17–19). These modified promoters demonstrate greatly increased androgen inducibility while maintaining tissue specificity in vitro or in vivo.

The RPB promoter was initially identified as one of several prostate-specific genes (20). Although its function is still unclear, RPB is a member of the lipocalin superfamily and is found in both the nucleus of prostate epithelial cells and in prostatic secretions (21). Cooperative binding of AR to two distinct AR-binding sites, AR binding site-1 (nucleotides −236 to −223) and AR binding site-2 (nucleotides −140 to −117), in an ARR is required for maximum androgen induction of gene expression (22, 23). However, the presence of one copy of the ARR of PB, as in the minimal PB promoter, although sufficient for developing animal models for prostate cancer (24), may be insufficient for suicide gene therapy. This concern led to the development of a small 500-bp composite promoter, ARR:PB, which contains two copies of the PB ARR (−244 to −96) upstream of the minimal promoter, leading to very strong AR-dependent expression (17).
Although promoter strength may be an important criterion for cytotoxic gene therapy, the choice of suicide gene may be equally important. For example, high-level expression of HSV-tk or bacterial proteins, such as nitroreductase, may still have limited efficacy or safety. Because HSV-tk converts ganciclovir (or other analogues) to a DNA-terminating drug (25), its primary utility is against rapidly dividing cells. In contrast, prostate adenocarcinomas grow slowly (26), undergoing limited HSV-tk efficacy (27). Moreover, suicide genes that are relatively cell cycle independent, such as nitroreductase or purine nucleoside phosphorylase, convert a relatively benign prodrug (e.g., CB1954 and 6-mercaptopurine-2′-deoxycytoside) to a highly toxic and membrane permeable mutagenic agent (reviewed in Ref. 28), underlying their narrow therapeutic index.

In contrast, we have been developing inducible caspases as a novel suicide gene therapy strategy for prostate and other forms of cancer (29–31). These are based on using lipid-permeable dimeric drug CIDs (32) to cross-link genetically modified procaspases containing an NH2-terminal CID-binding domain, leading to caspase transproteolysis and activation in the presence of CID, resulting in cellular apoptosis. This novel binary system allows the potential for more stringent control over caspase activation than simple overexpression of native caspases has allowed (33). We have demonstrated previously that iCaspases can kill prostate cells in vitro and in vivo, leading to significant decreases in tumor size and growth in a murine model (31). However, these studies used a CMV-based promoter to drive transgene expression. Despite mechanical targeting of these vectors, subsequent studies by those of others and us have demonstrated nontargeted transgene expression and low-level toxicity in distant organ sites, such as the liver and brain (19, 27, 34).

Thus, to further improve the safety and efficacy of gene therapy for prostate cancer, we have designed a nonreplicating adenoviral vector that incorporates the synthetic ligand iCaspase-9 under the transcriptional control of ARR2 PB for the treatment of prostate cancer. iCaspase-9 is a nonmutagenic ADS that can trigger apoptosis in dividing and nondividing cells with fast kinetics and low basal activity (30). Here, we describe two novel helper-dependent ADVs under the transcriptional control of ARR2 PB for the treatment of prostate cancer. iCaspase-9 is a nonmutagenic ADS that can trigger apoptosis in dividing and nondividing cells with fast kinetics and low basal activity (30). Here, we describe two novel helper-dependent ADVs under the transcriptional control of ARR2 PB, ADV.ARR2 PB-iCasp9 expressing iCaspase-9 and control reporter virus ADV.ARR2 PB-EGFP expressing EGFP. We demonstrate that ADV.ARR2 PB maintains its prostate tissue specificity in the context of an ADV vector, targeting EGFP or iCaspase-9 expression specifically to AR+ LNCaP cells. Furthermore, we show that ADV.ARR2 PB-iCasp9 can cause CID-dependent apoptosis of LNCaP cell lines but not other AR− cell lines, in vitro and in vivo, and can lead to significant size reductions of xenograft tumors and greatly extended animal survival.

**MATERIALS AND METHODS**

**Cell Lines**

Androgen-sensitive human prostate adenocarcinoma cell line LNCaP, androgen-independent prostate cancer cell line PC-3, and non-prostate cancer cell lines 293, HeLa (cervical cancer), and U87 (glioma) were obtained from the American Type Culture Collection (Rockville, MD). LNCaP, PC-3, MCF-7, and MDA231 cells were maintained in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin (Life Technologies, Inc.). U87 cells were grown in MEM (Life Technologies, Inc.), 10% FBS, penicillin, and streptomycin. HuH-7, 293, and HeLa cells were grown in DMEM (Life Technologies, Inc.), 10% FBS, penicillin, and streptomycin. Charcoal/dextran-treated FBS (Cyclone, Logan, UT) was substituted for general FBS in androgen inducibility experiments.

**Plasmid Constructs**

A 500-bp ARR2 PB fragment was released from pSK/ARR2 PB (17) by digestion with Clal/Xbal and inserted into Clal/Xbal-digested pSh1/hk2-E/P-SEAP (19) to give pSh1/ARR2 PB-SEAP. The resulting pSh1/ARR2 PB-SEAP was digested with HindIII and religated to obtain pSh1/ARR2 PB-SEAP, containing PB sequences from −286 to +28 relative to the cap site. Treatment of promoter/enhancer elements were excised from pB/PL/PSA-CAT and used to replace hK2 elements in pSh1/hk2-E/P-SEAP. PSA-E2/P-SEAP contains two tandem copies of the human PSA enhancer [−3522 (XbaI site) to −3874 (Psp1406d site)] upstream of the promoter fragment (+541 to +86 (HindIII site)], pSh1/kSP-SEAP was derived from pSh1/PSA-E2/P-SEAP after Asp718/XbaI digestion, klenow-blunting, and religation. pSh1/hk2/E3/P-SEAP, pCMV-SEAP (19), and pSh1/SFip6/hk2Fip6-Casp9 were described previously (30).

**Transfection and SEAP Assay**

Cells were seeded in six-well plates at 40–50% confluence in complete medium 18 h prior to transfection. We used FuGENE6 (Roche, Indianapolis, IN) to transfect the designated plasmids, according to the manufacturer’s instructions. The plasmid DNA:FuGENE6 ratio was optimized for each cell type and varied from 1 to 2 μg of DNA per 3 μl of FuGENE6 reagent. For promoter comparisons, the same molar amount of plasmid DNA was used per transfection. Unmodified expression vector, pSh1, was used as a background control, and promoterless pSh1/ΔSEAP was used as a negative control. After a 5-h incubation with DNA/FuGENE6, standard growth medium was replaced with medium containing 10% charcoal/dextran-treated FBS in the presence, or absence, of synthetic androgen (R1881; NEN/DuPont). After 48 h, supernatants were harvested and assayed for SEAP activity as described previously (32). Units of SEAP activity were expressed as means of triplicate transfections, which were repeated at least three times.

**Construction of ADV Vectors**

**Adenoviral Shuttle Vectors.** The 530-bp KpnI/SpeI ARR2 PB fragment released from pSK/ARR2 PB was inserted into similar sites of pAdloxΔ/hk2-E/P-iCasp9 to replace the hK2/E/P-CID promoter to give pAdloxΔ/ARR2 PB-iCasp9. The PNP-IREs NcoI fragment from pAdloxΔ/ARR2 PB-PNP-IREsEGFP4 was removed to give pAdloxΔ/ARR2 PB-EGFP.

**Generation of Recombinant ADVs.** The Cre-lox system for construction of ADV vectors has been used as described previously (35). Briefly, CRE 8 cells were plated onto six-well plates 18 h before transfection. Shuttle vectors pAdloxΔ/ARR2 PB-iCasp9 or pAdloxΔ/ARR2 PB-EGFP were cotransfected with ARR2 PB-iCasp9 or pAdloxΔ/ARR2 PB-EGFP expressing EGFP. We demonstrate that ADV.ARR2 PB maintains its prostate tissue specificity in the context of an ADV vector, targeting EGFP or iCaspase-9 expression specifically to AR+ LNCaP cells. Furthermore, we show that ADV.ARR2 PB-iCasp9 can cause CID-dependent apoptosis of LNCaP cell lines but not other AR− cell lines, in vitro and in vivo, and can lead to significant size reductions of xenograft tumors and greatly extended animal survival.

**Western Blotting**

LNCaP, PC-3, and HuH-7 cells were seeded in six-well plates 18 h before infection. Cells were infected with ADV.ARR2 PB-iCasp9 at a MOI of 10. Eighteen h after infection, cells were treated with R1881, AP20187 (CID), R1881/AP20187, or control PBS. At 1, 2, 4, and 8 h after treatment, protein lysates were prepared with RIPA buffer [10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS] 1:100 protease inhibitor mixture (Sigma Chemical Co., St. Louis, MO) at 0°C for 30 min. Cell debris was removed by centrifugation, and supernatants were boiled in 1:1 Laemmli sample buffer (Bio-Rad, Hercules, CA) with 5% 2-mercaptoethanol. Five μg of total protein per lane were separated on 12% SDS-PAGE.
gels. Membranes were probed with monoclonal anti-HA antibody HA.11 (BABCO, Richmond, CA) or monoclonal anti-α-tubulin (Ab-1; Calbiochem), followed by polymodal horseradish peroxidase-conjugated goat antimouse antibody (Bio-Rad). Bands were detected with SuperSignal chemiluminescent substrate (Pierce, Rockford, IL).

**Specific Expression of EGFP in Vitro**

Cells were transferred to six-well plates at 50% confluence in complete medium 1 day before infection. Cells were infected with CsCl-purified ADV at various MOIs (1, 10, and 50) as described previously (36). Four days after infection, cells were photographed using a fluorescence microscope.

**Cytotoxicity Assay**

Cells were placed on six-well plates prior to infection as above. At ~70% confluence, cells were incubated with ADV.ARR.PB-iCasp9 or ADV.ARR.PB-EGFP (MOI, 10). After 18 h, cells were stimulated with 10 nM R1881 or PBS. After 24 h, cells were treated with log dilutions of AP20187. After a final 24-h incubation, viable cells were counted by trypan blue dye exclusion.

**Apoptosis Assay**

To identify cells undergoing apoptosis after either in *vivo* or in *vitro* activation of iCaspase-9, an in situ TUNEL assay was performed according to the manufacturer’s (Roche Boehringer Mannheim, Mannheim, Germany) instructions. For the in *vivo* study, 16 h after ADV infection, cells were treated with R1881 (10 nM), AP20187 (10 nM), both, or neither for 8 h. Cells were then rinsed with PBS and fixed with 4% paraformaldehyde for 1 h at room temperature. After rinsing with PBS, cells were incubated in ice-cold permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min at 0°C. Cells were rinsed with PBS and stained with TUNEL reaction mixture for 60 min at 37°C. After another PBS wash, cells were incubated with Converter-AP for 30 min at 37°C. Cells were rinsed and incubated with substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Life Technologies, Inc.) for 30 min. After a final PBS rinse (repeated twice), cells were microphotographed. For the in *vitro* study, frozen tumor samples were sectioned with a cryostat and stained as described above.

**Animal Model**

Athymic male BALB/c nu/nu mice (National Cancer Institute, Frederick, MD), 6–8 weeks of age, were used as hosts for human xenografts. Mice were maintained in a specific pathogen-free environment, in compliance with Baylor College of Medicine policy. Cells in logarithmic-phase growth were lightly trypsinized and washed twice with OPTI medium (Life Technologies, Inc.). HuH-7 cell suspensions (1 × 10⁶ cells/200 μl) were s.c. injected into the right flank of the mice. To establish LNCaP tumors, a 1:4 mix of 100 μl of cell suspension (1 × 10⁶ cells) with Matrigel (Becton Dickinson, Bedford, MA) was injected. When tumors reached ~70 mm³, the designated virus was injected intramuscularly. Five days later, mice were injected i.p. with AP20187 at 2 mg/kg for two consecutive days. To test for apoptosis in the tumor xenografts, mice were sacrificed 2 days after injection, and tumor, liver, and brain were excised, frozen, and sectioned for apoptosis detection by TUNEL assay and H&E staining. To monitor tumor growth, tumors were measured biweekly with vernier calipers. Volumes were calculated using the formula, \( V = \frac{L \times W^2 \times 0.5236}{2} \) (where \( L \) is tumor length and \( W \) is width). Mice with excessive tumor burdens (i.e., \( L > 20 \text{ mm} \) or \( W > 15 \text{ mm} \)) or appearing in distress (i.e., labored breathing, cachectic, and lethargic, among others) were euthanized by humane standards.

**RESULTS**

**Strength and Androgen Specificity of Multiple Composite Prostate-directed Promoters.** In addition to recently described PSA and hK2-based prostate-specific composite promoters PSA.EEP (18) and hK2.E3/P (19), respectively, a smaller composite promoter, ARR.PB, based on the rat PB promoter has been described recently (17). To guide our selection of the ideal prostate-specific (or “directed”) promoter for restricted, high-level delivery of therapeutic ADSs, we compared the activity and androgen inducibility of a minimal rPB promoter, ARR.PB, (–286 to +24), with ARR.PB, containing an additional copy of the ARR (–244 to –96; Ref. 37). Although the minimal promoter was only weakly induced by nonmetabolizable androgen analogue R1881 (1.0 nM) in AR-responsive LNCaP cells, ARR.PB was induced nearly 1000-fold, consistent with the initial characterization of ARR.PB (17). Similar results were achieved in an AR+ variant of PC-3 prostate cancer cells, designated PC-3-AR, whereas R1881-induced reporter expression was barely detectable in parental AR−/− PC-3 cells (data not shown). Thus, all three composite promoters, PSA.EEP, hK2.E3/P, and ARR.PB, are AR-dependent and comparable with the very strong “universal” CMV enhancer/promoter, demonstrating that very strong androgen inducibility is possible in a compact E/P.

Although most non-prostate cells lack AR expression because of the degeneracy of some steroid-binding elements, we investigated the stringency of PSA, hK2, and PB-based composite promoters for androgen. In these studies, we compared the 3.5-kb PSA-E2/P E/P (based on PSA.EEP (18)), the 4-kb hK2.E3/P E/P (19), and the 500-bp ARR.PB E/P. LNCaP cells, PC-3-AR, and AR MCF-7 breast adenocarcinoma-derived cells were transfected with pSH1/ARR.PB-SEAP, pSH1/hK2.E3/P-SEAP, pSH1/PSA-E2/P-SEAP, or pCMV-SEAP reporter plasmids. Twenty-four h after transfection, cell aliquots were incubated with 1 nM R1881, 10 nM 17β-estradiol, 10 nM progesterone, 10 nM dexamethasone, or control diluent (0.1% ethanol). In PC-3-AR cells containing unmutated AR, only R1881 could activate the three androgen-inducible promoters (Fig. 1A). In contrast, in LNCaP cells containing the A877 mutation, found commonly in metastatic disease and associated with relaxed ligand specificity (38), the hK2 and PSA-derived promoters could also be induced by estrogen, progesterone, and glucocorticoid (Fig. 1B). However, the ARR.PB promoter could only be induced by R1881, suggesting that cts-binding elements adjacent to AREs or minor sequence differences within AREs can have marked effects on steroid specificity. Furthermore, all three promoters could be induced by dexamethasone in GR+/− MCF-7 cells (Fig. 1C). These results further demonstrate that ARR.PB is androgen and AR dependent and is likely to act as a highly prostate-restricted promoter for gene therapy applications.

**Caspase 9-based ADS Triggers Apoptosis with Rapid Kinetics in Human Cells.** We previously developed a panel of inducible suicide genes based on Fas and Fas signaling intermediates, such as Fas-associated death domain, Apaf-1, and caspases 1, 3, 8, and 9 (29, 30, 39). These ADSs were based on the well-established observations that clustering of signaling molecules can often lead to their activation and that small lipid-permeable dimeric ligands, CIDs (32, 40), can be used to induce this clustering if target molecules are modified to contain one or more ligand-binding domains. A CID-inducible caspase-9, iCaspase-9 (Fig. 2A), proved to be the most CID-sensitive ADS in Jurkat T cells (30). iCaspase-9 was made by genetically fusing two CID-binding domains, Fv and Fv, derived from FK506-binding protein, to the NH₂ terminus of caspase-9. Fv contains a Phe-36 to Val substitution to accommodate the high-sensitivity CID, AP20187 (Kd, ~0.1 nm; Ref. 41), Fv contains wobble bp substitutions to maintain genetic integrity and eliminate homologous recombination (42), and Fv, contains a short Gly-Ser linker to increase flexibility (30). When CID binds to the Fv, pro-iCaspase-9 clustering ensues, leading to transproteolysis, rearrangement, and full caspase activation.

To evaluate whether iCaspase-9 could trigger apoptosis in other tumor lines, plasmid pSH1/Sq.E-Fv-iCasp9 was cotransfected along with reporter plasmid pCMV-SEAP into LNCaP, PC-3, HuH-7, and HeLa cells. Sixteen h later, cells were stimulated with 100 nM AP20187 or were untreated. After an additional 24 h, supernatants containing the soluble SEAP reporter were assayed in a chemiluminescent assay following transproteolysis.
were harvested and assayed for SEAP reporter activity. In each case, dimerization of caspase-9 led to a strong diminution of reporter activity. Residual reporter activity may reflect the lag time to turn off transcription or preexisting reporter protein. Because a diminution in reporter activity should reflect a reduction in cell viability, the data strongly suggest that iCaspase-9 can trigger significant, if not complete, apoptosis of LNCaP (2582 versus 1409 units), HuH-7 (5348 versus 1293 units), and HeLa (4500 versus 1311 units) cells; however, without R1881, EGFP was only weakly detectable in 4% of LNCaP cells. These results strongly indicate that ARR2 PB is able to maintain its androgen and AR specificity in the context of an ADV vector.

Prostate Adenocarcinoma-specific Cytotoxicity of iCaspase-9 Expressing ADV Vector. To further investigate the possibility of creating a viral vector triggering prostate-specific apoptosis, we generated recombinant ADV, ADV.ARR2PB-iCasp9, expressing iCaspase-9 under the transcriptional control of ARR_PB (Fig. 3B). Initially, LNCaP cells were infected with either ADV.ARR2PB-iCasp9 or ADV.CMV-EGFP at various MOIs (0.2–100). After overnight incubation with virus, cells were treated with R1881 (1 nM), CID AP20187 (100 nM), both reagents, or neither. After 24 additional h, cell viability was determined by trypan blue dye exclusion relative to control mock-infected cells. The results showed that after treatment with R1881 and AP20187, cell survival was 28, 8, and ~0% at MOIs

![Fig. 1. Steroid dependence of various AR-binding composite E/Ps. A, the variant PC-3 line, PC-3-AR, ectopically expressing wild-type human AR was transfected with reporter plasmids containing the indicated promoters derived from prostate-specific genes, hK2, PSA/hK3, and pRB, or CMV. Cells were treated as above with either R1881 (1 nM), 17-β-estradiol (10 nM), progesterone (10 nM), dexamethasone (10 nM), or control diluent. After 48 h, supernatants were assayed for SEAP activity. LNCaP cells expressing mutant AR (B) or MCF-7 cells expressing GR but no AR (C) were transfected and analyzed as above. Data are representative of two independent experiments performed in triplicate; bars, SD.](image-url)

![Fig. 2. Cell killing by CID-inducible iCaspase-9. A, model of iCaspase-9. Transmembrane diffusion of CID (e.g., AP20187) induces the cross-linking of CID-binding FK506-binding proteins (e.g., Fv, light speckle; Fvls, dark speckle) fused with pro-caspase-9 (stripes), leading to transproteolysis and processing of pro-caspase-9 to active caspase-9 and ultimately apoptosis. B, plasmid pSH1/SN-E-Fv-Casp9 was transiently transfected into LNCaP, PC-3, HuH-7, and HeLa cells along with constitutively expressed reporter plasmid pCMV-SEAP. Twenty-four h after transfection, medium was replaced with fresh medium containing AP20187 (100 nM) or CID diluent. After an additional 24-h incubation, supernatants were harvested for SEAP analysis. Data are representative of four independent experiments in triplicate; bars, SD.](image-url)

![Fig. 3. Construction of recombinant ADVs. A, ADV.ARR2PB-EGFP expresses EGFP under the transcriptional control of ARR_PB in the E1 site of ΔE1/ΔE3 helper-dependent ADV, Psi, constructed by the method of Hardy et al. (35). B, similarly, ADV.ARR2PB-iCasp9 expresses E-Fv-Casp9-E rather than EGFP. E, influenza-derived hemagglutinin epitope tag; pA, SV40 poly(A) sequence.](image-url)
of 2, 10, and ≥25, respectively (Fig. 5A). In contrast, ADV.CMV-EGFP had little effect on cell viability, even at an MOI of 100.

To determine whether LNCaP cells were specifically killed by ADV.ARR2 PB-iCasp9, a panel of cells was infected with ADV.ARR2 PB-iCasp9 or ADV.ARR2 PB-EGFP at an MOI of 25. Cells infected with mock virus acted as a control. Virus-infected cells were treated essentially as described above except that the MOI was held constant and AP20187 was varied. As before, LNCaP cells were killed if infected with ADV.ARR2 PB-iCasp9 and cotreated with R1881 and AP20187 (Fig. 5). Moreover, the IC50 CID was astonishingly low, ~3 pm. In contrast, ADV.ARR2 PB-iCasp9 conferred no detectable toxicity to AR− PC-3 cells or non-prostate cell lines HuH-7, U87, MDA231, and HeLa, even at 100 nm AP20187 and a MOI of 100 (not shown). Furthermore, ADV.ARR2 PB-EGFP conferred no observable toxicity to any cell lines tested in this assay. Thus, ADV.ARR2 PB-iCasp9 can trigger cell death in an androgen-, CID-, and tissue-specific manner.

To further characterize the kinetics of induction and killing by iCaspase-9, cellular extracts from ADV.ARR2 PB-iCasp9-transduced cells were analyzed by Western blot for iCaspase-9 expression and processing. Unprocessed pro-iCaspses-9 was detectable within 1 h of R1881 (1 nm) administration to ADV-transduced (MOI, 10) LNCaP cells and continued to increase for at least 8 h (Fig. 6). When cells were also treated with AP20187 (100 nm), pro-iCaspase-9 levels were significantly reduced, and the smaller processed active caspase-9 became apparent. However, the increase in active caspase-9 could not fully account for the reduction of pro-iCaspase-9 because of the possible instability of active caspase-9 and the likelihood that cellular apoptosis was occurring. As expected, incubation of transduced cells with CID alone did not induce the transgene. Also, transgene expression was not androgen-inducible in AR− cells, although these cells were easily transduced with control ADV.CMV-EGFP, as indicated by comparable transgene levels to transduced LNCaP cells (Fig. 4 and not shown).
ADV.ARR2 PB-iCasp9 could be maintained with either LNCaP or HuH-7 tumor cells mixed with the extra-mice. Six- to eight-week-old BALB/c xenograft LNCaP and HuH-7 tumor models in immunodeficient male mice were injected intratumorally with 1 x 10^9 pfu of ADV.ARR2 PB-iCasp9 or ADV.ARR2 PB-EGFP at a MOI of 10. Sixteen h later, cells were stimulated with R1881 (1 nM) for 24 h and then were treated with log dilutions of AP20187. After an additional 24-h incubation, cell viability was determined by trypan blue staining as above. The results represent the mean of three independent experiments. 

Consistent with expression and processing of pro-iCaspase-9 after R1881/AP20187 treatment, ADV.ARR2 PB-iCasp9-transduced LNCaP cells were rounded up and condensed, indicative of apoptotic death (Fig. 7A). TUNEL analysis confirmed that all of these morphologically apoptotic LNCaP cells also had fragmented terminal deoxynucleotidyl transferase-reactive DNA (Fig. 7B). Thus, ADV.ARR2 PB-iCasp9 transduction can lead to prostate-specific apoptosis in an androgen- and CID-dependent manner.

**ADV.ARR2 PB-iCasp9/CID-mediated Regression of LNCaP Tumors in Vivo.** To further explore whether tissue-specific killing by ADV.ARR2 PB-iCasp9 could be maintained in vivo, we established xenograft LNCaP and HuH-7 tumor models in immunodeficient male mice. Six- to eight-week-old BALB/c nu/nu mice were injected s.c. with either 10^6 LNCaP or HuH-7 tumor cells mixed with the extracellular matrix-derived graft enhancer, Matrigel. After ~4 weeks, 50–75 mm^3 tumors were injected intratumorally with 1 x 10^5 pfu of either ADV.ARR2 PB-iCasp9 or ADV.CMV-EGFP. Five days after injection, mice were injected i.p. with AP20187. Two days later, mice were euthanized, and tumors were excised and cryogenically sectioned. Although we observed no obvious toxicity to any animals from these treatments, TUNEL analyses revealed clear and widespread apoptosis in ADV.ARR2 PB-iCasp9-transduced, AP20187-treated LNCaP-derived tumors in vivo (Fig. 8). Greatly reduced but still detectable apoptosis was also seen in the absence of AP20187. However, apoptosis was not detectable in ADV.CMV-EGFP-transduced cells or in any of the HuH-7-transduced tumors. Thus, ADV.ARR2 PB-iCasp9 can trigger efficient prostate-specific apoptosis in vivo at physiological levels of circulating androgen.

Because expression and activation of iCaspase-9 could efficiently induce apoptosis in s.c. LNCaP tumors in a male nude mouse xenograft model, we further evaluated whether iCaspase-9 treatment could inhibit LNCaP tumor growth. These s.c. tumors were 58–98 (average, 71) mm^3 in volume, mice were divided into three equal groups (n = 7) with comparable tumor ranges and were injected intratumorally with 1 x 10^5 pfu of ADV.ARR2 PB-iCasp9 (two groups) or control virus, ADV.ARR2 PB-EGFP. After 5 days, mice bearing ADV.ARR2 PB-iCaspase-9-treated tumors were randomly split again into two groups. One group received AP20187 (2 mg/kg) for 2 consecutive days, and one group received CID diluent alone.

To further reduce any nonspecific confounding effects of AP20187 (although this has never been seen before in vivo at this dose), ADV.ARR2 PB-EGFP transduced control mice were also given AP20187. Although the growth of ADV.ARR2 PB-EGFP-transduced tumors was indistinguishable from non-CID-treated ADV.ARR2 PB-iCaspase-9-transduced tumors, all (seven of seven) of the LNCaP tumors regressed after treatment with ADV.ARR2 PB-iCaspase 9 and AP20187 (Fig. 9). Furthermore, two of these animals had no detectable tumors after treatment up to 6 months after virus injection. Four mice within the full treatment group initially had very small, slow-growing residual tumors (<100 mm^3) until day 53, whereas only one tumor, which was the largest of the group at the beginning of treatment, grew at a rapid rate and led to euthanasia at day 63. Similar results were achieved in two pilot studies (not shown). Moreover, tumor-bearing animals treated with ADV.ARR2 PB-iCaspase 9 and AP20187 had significantly prolonged survival, with four of seven mice surviving to the termination point of the experiment at day 180. In contrast, the AP20187/EGFP and non-AP20187/iCaspase-9-treated control mice died at mean times of 51.6 ± 4.7 and 51.0 ± 5.4 days, respectively.
after virus injection. Thus, tissue-specific ablation of porous tumors is possible using ADV-mediated transfer of iCaspase-9 with subsequent CID-mediated caspase induction.

**DISCUSSION**

Multiple previous studies have reflected the deleterious effects associated with dissemination of intratumorally injected non-tissue-specific ADV. In preclinical studies evaluating treatment of various tumor models, hepatotoxicity was observed after administration of ADV expressing CMV promoter-driven HSV-tk and ganciclovir (43, 44). These findings are consistent with our observation of ectopic transgene expression in liver and brain tissue after intratumoral injection of ADV expressing CMV-directed EGFP (19). Despite strategies to target viral vectors to tumor tissue, there is little evidence that this has been achieved efficiently in vivo. However, because the prostate is an encapsulated organ that is easily accessible and imaged either transrectally or transperineally, efficient delivery of genes to the prostate organ should be easily accomplished. Nevertheless, despite intraprostatic injection of HSV-tk expressing ADV in prostate cancer patients, Herman et al. (45) has reported that 4 of 18 patients exhibited grade 1–2 toxicity, and 1 patient experienced grade 4 thrombocytopenia and grade 3 hepatotoxicity (45). Thus, without further confining expression to prostate epithelial, or stromal cells, systemic release of infectious vector could still lead to systemic toxicity. Therefore, to further restrict gene expression to targeted prostate cells, we and several groups have used prostate-specific transcriptional elements (reviewed in Ref. 46).

In the present study, we confirm that the relatively compact 468-bp ARR 2 PB is a powerful, prostate adenocarcinoma-specific, androgen-regulated promoter and demonstrate this tissue specificity in the context of an ADV vector. Our data have shown that the activity of ARR2 PB is 764-fold higher than a promoterless control plasmid in LNCaP cells after R1881 induction, comparable with the universal CMV E/P (Fig. 1). Although androgen-induced ARR2 PB activity is very strong in LNCaP cells after R1881 induction, comparable with the universal CMV E/P (Fig. 1). Although androgen-induced ARR2 PB activity is very strong in LNCaP cells, its activity is much lower in the non-AR-producing prostate cell line PC-3 cells and in several nonprostatic cell lines (Fig. 5). Use of cis-acting insulator sequences could further improve fidelity (47). Furthermore, we demonstrate that the ARR2 PB specific ADV.
promoter may be an ideal promoter for prostate-specific expression of proapoptotic suicide genes.

Apoptosis resistance may enhance the malignant properties of prostate cancer cells, contributing to metastasis (48) and androgen-independent disease progression (49). To overcome apoptosis resistance, a number of proapoptotic molecules have been overexpressed in prostate cancer cells, including p53 (50), Fas ligand (51), caspase-7 (33), Bax (52), PHyde (7), and p16 (53). However, the efficacy of these approaches have been limited for numerous reasons: (a) only a subset of prostate cancer cells have p53 mutations and mutations downstream of p53 could limit its utility; (b) the Fas-mediated death pathway may be blocked by antiapoptotic proteins, such as c-FLIP (54), Bcl-2 family members (55), and a variety of caspase-directed inhibitors of apoptosis (56); and (c) sufficient levels of proapoptotic molecules to trigger apoptosis may be difficult to achieve (57). In contrast, CID-mediated aggregation and activation of caspases and other proapoptotic molecules can extend the “radius of death” to cells expressing subthreshold levels of these (29–31, 39, 58–61). Furthermore, we have optimized the CID inducibility of several ADSs so that subnanomolar activation by third generation CIDs (e.g., AP20187) is achieved (30). Therefore, although tumor cells may express antiapoptotic molecules, all cell lines tested thus far were sensitive to these optimized inducible caspases (Refs. 30 and 31; Fig. 4). Thus, tissue-specific targeting of iCaspase-9 (and possibly caspases 1, 3, and 7) may be effective at triggering apoptosis in most, if not all, tumor cells.

Furthermore, we demonstrate that ARR,PB-targeted iCaspase-9, based on CID technology, can be used to ablate LNCaP cells in an R1881- and AP20187-dependent manner with speed, efficiency, and sensitivity; <24 h was required after CID treatment (IC50 ~3 pm) to kill 28, 8, and ~0% of LNCaP cells at MOIs of 2, 10, and 25, respectively (Fig. 5). In vivo experiments demonstrated that intratumoral injection of ADV.ARR,PB-iCas9 could specifically induce apoptosis of xenograft LNCaP tumors in a primarily AP20187-dependent manner and could significantly suppress LNCaP tumor growth and prolong life span after a single administration of AP20187 (Figs. 8 and 9).

Compared with other well-characterized suicide gene/prodrug strategies, such as HSV-tk/ganciclovir, cytotoxic deaminase/5-fluorocytosine, and nitroreductase/CB1954, the iCaspase system has several distinct advantages for prostate gene therapy including: (a) cell cycle independence (an important parameter for efficacy against slow-growing prostate cancer or benign prostate hyperplasias); (b) convenience (CIDs are nontoxic, lipid-permeable ligands and could be administered either p.o. or in one or two i.v. injections as opposed to multiple daily injections over 5–14 days that is required for other prodrugs); and (c) immunogenicity [apoptotic tumor cells are efficiently phagocytosed by antigen-presenting cells (62)]. Moreover, compared with suicide genes that activate a relatively inert prodrug to a highly toxic, and typically mutagenic, chemotherapeutic drug, iCaspases are activated by otherwise benign dimerizing drugs, leading to cellular apoptosis in the absence of mutagenesis or detectable bystander killing (31). Although bystander killing has been considered an attribute of traditional GDEPT therapy in which tumor targeting is often inefficient, the local release of activated prodrugs would also cause a commensurate increase in local toxicity among nontargeted cells.

We demonstrated previously significant but limited tumor regression after treatment with CID and ADV expressing iCaspase-1 directed by the promiscuous CMV promoter in s.c. murine TRAMP-C2 tumors (31). In that report, we observed limited distribution of virus confined to the area immediately adjacent to injection sites. In contrast, in this study, we demonstrated profound tumor regression and complete tumor eradication in some hosts using the human LNCaP xenograft model. This more dramatic response may be attributable to the fact that these tumors were less dense, without the prominent extracellular matrix associated with TRAMP-C2 tumors (63), allowing a wider distribution of virus. Furthermore, Matrigel, which was mixed with LNCaP cells at a 4:1 ratio during the establishment of these tumors, may have acted as a reservoir of virus, enhancing the extent of infection as has been seen with other artificial matrices (64).

Although we have clearly demonstrated efficacy in AR-positive human prostate tumor cells, one concern may be the broad utility of an androgen-responsive promoter to drive expression of iCaspase-9 for the treatment of prostate cancer. Most patients with clinically localized prostate cancer are currently treated with either surgery or radiation therapy, with hormonal ablation therapy typically reserved...
for patients with more advanced disease. Studies evaluating the role of neoadjuvant hormonal therapy to “downstage” patients undergoing radical prostatectomy have been, for the most part, disappointing (65). Hormonal therapy may be used transiently to decrease the prostate volume in patients undergoing brachytherapy. However, most patients with localized prostate cancer are not currently treated with androgen ablation, allowing the use of this vector for prostate cancer therapy.

For patients with advanced, metastatic prostate cancer, hormonal ablation is the primary choice of therapy. However, after chemical castration, the adrenal glands secrete large amounts of the inactive precursor steroids dehydroepiandrosterone and androstenedione as a compensatory source of androgen (66). Furthermore, most androgen-independent prostate adenocarcinomas cells up-regulate AR expression, select for mutant AR with increased steroid responsiveness or reduced steroid specificity, or up-regulate growth factor signaling that can stimulate AR activity (reviewed in Ref. 67). Moreover, the significant side effects associated with this therapy have led to the more widespread use of intermittent ablation protocols, in which patients are treated with leutinizing hormone releasing hormone agonists for up to 12 months before discontinuing therapy until tumor progression begins to occur (68). Thus, for numerous disease stages, patients are often hormonally intact, allowing the use of androgen-responsive vectors to direct expression of therapeutic genes to prostate tissue.

In summary, we have developed a novel prostate cancer-specific iCASpase-9 gene therapy strategy based on transcriptional targeting of a CID-inducible caspase-9 vector. Treatment with the ADV.ARR_pbICasp-9 vector caused extensive apoptosis throughout LNCaP xenograft tumors associated with significant tumor regression and growth suppression. Although we have mainly focused on the ARR_PB promoter in this study, we have compared three strong androgen-inducible promoters (Fig. 1). The relative advantages or disadvantages of these and other promoters may not be clear until they are tested in fresh prostate cancer explants or in clinical trials. This study demonstrates the feasibility of prostate-specific iCASpase expression for prostate cancer gene therapy.

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REFERENCES

Adenovirus-mediated Tissue-targeted Expression of a Caspase-9-based Artificial Death Switch for the Treatment of Prostate Cancer

Xiaoming Xie, Xiuqin Zhao, Yuanfang Liu, et al.

Cancer Res 2001;61:6795-6804.

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