Adenovirus-mediated Transfer of Inducible Caspases: A Novel “Death Switch”
Gene Therapeutic Approach to Prostate Cancer

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ABSTRACT

In patients with localized prostate cancer, radical prostatectomy and radiation therapy, although effective in controlling localized disease, are often associated with significant side effects attributable to injury of adjacent tissues. Moreover, patients with metastatic disease eventually fail systemic hormonal or chemotherapy because of the development of progressive, refractory disease. In this study, we evaluated the safety and efficacy of a novel suicide gene therapy that could potentially spare normal tissue while bypassing molecular mechanisms of apoptosis resistance by using chemically inducible effector caspases to trigger apoptosis in prostate cancer cells. Initially, we compared the ability of a panel of inducible Fas signaling intermediates to kill human and murine prostate cancer cell lines. On the basis of the superior killing by downstream caspase-1 and caspase-3, replication-deficient adenoviral vectors expressing conditional caspase-1 (Ad-G/iCasp1) or caspase-3 (Ad-G/iCasp3), regulated by nontoxic, lipid-permeable, chemical inducers of dimerization (CID), were constructed. Upon vector transduction followed by CID administration, aggregation and activation of these recombinant caspases occur, leading to rapid apoptosis. In vitro, both human (LNCaP and PC-3) and murine (TRAMP-C2 and TRAMP-C2G) prostate cancer cell lines were efficiently transduced and killed in a CID-dependent fashion. In vivo, direct injection of Ad-G/iCasp1 into s.c. TRAMP-C2 tumors caused focal but extensive apoptosis without evidence for a bystander effect at the maximal viral dose (i.e., 2.5 × 10^10 viral particles/25 μl) in host animals that also received CID compared with control animals. Treatment with Ad-G/iCasp1 plus CID resulted in a transient, yet significant, reduction both in tumor growth and volume compared with tumors treated with vector but not CID (P < 0.035) or vector-diluent plus CID (P < 0.022), both of which grew more rapidly. These results demonstrate that CID-regulated, caspase-based suicide gene therapy is safe and can inhibit the growth of experimental prostate cancer in vitro and in vivo through potent induction of apoptosis, providing a rationale for further development.

INTRODUCTION

Through the widespread use of prostate-specific antigen-based screening, the number of men diagnosed and treated for clinically localized prostate cancer over the past decade has dramatically increased (1). Concomitantly, the incidence of clinical metastatic disease at the time of initial diagnosis has dropped considerably, in concert with an overall decrease in prostate cancer mortality (2). Despite this favorable trend in prognosis, ~30% of patients treated for localized disease will relapse, as evidenced by a detectable or rising prostate-specific antigen, which is often attributable to early disseminated microscopic metastatic disease prior to primary therapy (3). The majority of these relapsing patients will eventually develop clinically evident metastatic disease (4), for which there is currently no available cure. Antiandrogen therapy and some chemotherapeutic agents typically lead initially to tumor regression, but the disease inevitably progresses as tumors develop resistance to these modalities. Furthermore, current prostate cancer therapies, either local or systemic, are associated with a significant negative impact on quality of life because of side effects (e.g., incontinence, impotence, rectal complications, loss of libido, hot flashes, and others) that are directly attributable to the therapy. Therefore, there is a clear need for new therapeutic approaches that can maintain or improve efficacy, reduce side effects, and address more effectively the problem of resistance to therapy and progression to metastatic disease.

One such potential novel approach toward prostate cancer is gene therapy. The unique features of the prostate, such as its expendability after the reproductive years, its easy accessibility through a transrectal or transurethral approach, and its encapsulated extraperitoneal location make it an especially suitable target for in situ gene therapy. The most extensively studied gene therapeutic approach to date for prostate cancer is the cytodestructive approach using a “suicide” gene therapy strategy. Suicide gene therapy typically involves the tumor-targeted delivery of genes encoding metabolic enzymes that convert systemically delivered, relatively innocuous prodrugs into highly toxic metabolites. The prototype for this approach is HSV-tk, which converts the deoxyinosinecicotide prodrug GCV into its phosphorylated form, leading to DNA chain termination and cell death (5). Other well-characterized suicide genes that convert relatively nontoxic prodrugs to highly cytotoxic agents include the Escherichia coli proteins cytosine deaminase, purine nucleoside phosphorylase, and nitroreductase. All of these suicide genes have demonstrated their ability to affect tumor regression through both direct and bystander mechanisms in murine models (6–10). However, the effectiveness of these strategies for human prostate cancer therapy may be blunted because of their more limited effect on slowly dividing cells that require prolonged expression of the therapeutic genes and long-term administration of the prodrugs to increase the proportion of cells affected (11). Furthermore, these genes can be inhibited by antiapoptotic proteins such as Bcl-2 or Bcl-x family members (12), which have been shown to be up-regulated in progressive, hormone-refractory prostate cancer (13). Finally, recent studies have found that suicide gene therapeutic approaches such as HSV-tk/GCV trigger apoptosis partially via the activation of FADD and caspase-8 (14), two upstream molecules in the Fas signaling pathway that have been shown to be blocked further downstream in a wide variety of cancers.

In previous reports, we described the development of a novel method for triggering apoptosis using engineered CID-regulated chimeric proteins based on endogenous proteins along the Fas signaling pathway. In this approach, target proteins are fused to one or more...
TREATMENT OF PROSTATE CANCER WITH INDUCTIBLE CASPASES

CID-binding domains, forcing noncovalent cross-linking by high-affinity interaction with CID (15), leading to protein aggregation and activation, propagating downstream signals. Here, we tested a panel of highly sensitive artificial death switches based on Fas and the Fas signaling intermediates FADD, caspase-1, caspase-3, and caspase-8 and found that multiple prostate tumor cell lines are insensitive to the upstream signaling molecules Fas, FADD, and caspase-8. In contrast, conditional alleles of caspase-1 and caspase-3, which act downstream of antiapoptotic proteins like Bcl-xL, triggered apoptosis in all cell lines tested.

Furthermore, we demonstrate for the first time the safety and efficacy of these chimeric inducible caspases for experimental prostate cancer therapy. Replication-deficient adenoviral vectors expressing inducible forms of caspase-1 (Ad-G/iCaspl) or caspase-3 (Ad-G/iCasps3) were constructed. In vitro, both, human and murine prostate cancer cell lines were efficiently transduced and rapidly killed by these novel vectors in a viral tier- and CID-dependent fashion. Additionally, experimental murine prostate tumors treated with Ad-G/iCaspl plus CID experienced a dramatic increase in apoptosis confined to virally transduced cells and consistent with a transient suppression of tumor growth. This study demonstrates a novel system for suicide gene therapy that is cell cycle independent, devoid of bystander cytotoxicity, and bypasses all known cellular antiapoptotic checkpoints that would potentially abrogate therapeutic efficacy.

MATERIALS AND METHODS

Prostate Cancer Cell Lines. Androgen-independent and -dependent prostate cancer cell lines (PC-3, Du145, and LNCaP) and 293 (a transformed human embryonic kidney cell line) were obtained from American Type Culture Collection (Rockville, MD). PC-3, Du145, Du145, and LNCaP cells were maintained in RPMI 1640 with 5% FBS. 293 cells were maintained in DMEM with 10% FBS. Mouse prostate cancer cell lines TRAMP-C2 (parental lines) and C2G (clonal derivative of C2), derived from the TRAMP model (16). TRAMP-C2 and TRAMP-C2G were grown in DMEM with 5% FBS and 5% NuSerum, supplemented with 10 nM DHT. TRAMP is a transgenic line of C57BL/6 mice that expresses SV40 early genes (T and T antigens) in a prostate-specific fashion under the transcriptional control of the minimal −426/+28 rat probasin promoter (17).

Transfections/Luciferase Assay. Human prostate cell lines, PC-3, Du145, and TSU-Prl, were plated at 1 × 105 cells/35-mm tissue culture dish. Twenty-four h later, cells that had reached 60–80% confluence were transfected with 1 µg of each expression plasmid containing a conditional Fas signaling intermediate plus 3 µg of the luciferase reporter plasmid, pGL2-Control (Promega). For all transfections, plasmid DNA mixed with FuGENE6 (Boehringer Mannheim), diluted in OPTI-MEM I (Life Technologies, Inc.) in a ratio of 2:3 of total DNA to FuGENE6, was added to cells for 24 h. After trypsinization, cells were split into two groups. One group was treated with 100 nM CID, either FK1012 (FasL constructs) or AP1903 (FasL), whereas the second group was treated with CID-diluent alone. After an additional 24 h, cells were lysed in 100 µl of Reporter Lysis Buffer (Promega). Ten-µl aliquots of each cell lysate were added to 90 µl of luciferase assay substrate solution, and reporter activity was measured using a Turner TD-20e luminometer. Luciferase activity is reported as the percentage of activity after CID addition relative to luciferase activity of mock-treated cells. Results shown are averages of at least three separate transfections ± SD.

Construction and Preparation of Adenoviral Vectors. Replication-deficient (ΔE1, E3) adenoviral vectors expressing conditional caspase-1 (Ad-G/iCaspl) or Caspase-3 (Ad-G/iCasps3) under the transcriptional control of the CMV promoter were constructed with the AdEasy System (Quantum Biotechnologies; Ref. 18). Initially, a 1.2-kb Norl-EcoRI fragment containing S-Fvls-Casp3-E or a 2.2-kb fragment containing S-EvFv-Fvls-Casp1 (19) was subcloned into Norl-EcoRV-digested pAdTrack-CMV to get shuttle vectors pAdTrack-CMV-Fvls-Casp3-E and pAdTrack-CMV-Fvls-Casp1, respectively. To generate recombinant adenoviral plasmids, shuttle vectors containing either CID-inducible caspase-1 or caspase-3 were linearized with Pmel, mixed at a 10:1 ratio with the adenoviral backbone plasmid, pAdEasy-1, and cotransfected (1 µg total) into E. coli strain BJ5138. Smaller kanamycin-resistant colonies, typically containing the large recombinant plasmids, were further analyzed by restriction site mapping. Replication-defective adenoviruses (Ad-GiCaspl and Ad-GiCasps3) were produced by transfection of 293 cells with a single isolate of each recombinant adenoviral vector. To reduce cytotoxicity from CID-independent caspase activation and increase titer, virus-transduced 293 cells were maintained in 50 mM pan-caspase inhibitor, Z-Asp-CH2-DCB (BACHEM). The recombinant adenoviruses were isolated from a single plaque, expanded in 293 cells, and purified by cesium gradient as described (18). Viral titers were initially determined by optical absorbance at 260 nm. Because these vectors also independently express enhanced GFP (Clontech) to facilitate the identification of cells successfully infected by virus, infectious particles were determined by titrating GFP-positive cells on 293 cells.

In Vitro Adenoviral Transduction and Cell-Killing Efficacy. Human (PC-3 and LNCaP) and murine (TRAMP-C2 and TRAMP-C2G) prostate cancer cell lines were plated in 24-well plates at 40,000 cells/well and mixed with increasing titers of medium-diluted Ad-GiCaspl or Ad-GiCasps3. Observation of GFP expression using a fluorescent microscope confirmed virus transduction and transgene expression. Twenty-four h after infection, 50 mM CID (e.g., AP20187) was added to the media for an additional 24 h. Medium containing nonadherent dead cells was removed, and the remaining living/ adherent cells were treated with the fixative glutaraldehyde (1%), stained with 0.5% crystal violet, and washed with distilled water. The crystal violet associated with fixed cells was dissolved in Sorenson’s solution and transferred to a 96-well plate. Absorbance was determined at 570 nm. Additionally, we assessed transduction efficiency and cell viability in these studies by dual-channel flow cytometry by using GFP fluorescence and propidium iodide exclusion, respectively.

Treatment of Experimental s.c. Tumors. C57BL/6 male mice (Harlan Laboratory, Houston, TX), 6–8 weeks of age, received s.c. injections of 4 × 106 TRAMP-C2 cells into the hind flank. Bidimensional tumor measurements were performed in a blinded fashion every second day with a Vernier caliper, and tumor volumes were calculated by the formula of a rotational ellipsoid (m1 2 × m2 × 0.5236, where m1 represents the shorter axis and m2 the longer axis). Three to four weeks after cell inoculation, when tumor volumes were ~50 mm3, recombinant Ad-GiCaspl suspended in 25 µl of vector-diluent (10 mM Tris (pH 7.5), 4% sucrose, and 2 mM MgCl2) or vector-diluent alone was injected directly into the tumor using a 30-gauge needle attached to a 100-µl syringe (Hamilton, Reno, Nevada). The needle was placed through an intradermal tract prior to tumor penetration to minimize leakage of virus after needle withdrawal. The needle bevel was then gently moved around within the tumor during injection to maximize the area exposed to virus. Depending on the experimental design, 2–6 days after virus injection, each animal was treated with one or two i.p. injections of either AP20187 (200 µg (16.7% 1,2-propanediol, 22.5% PEG400, and 1.25% Tween 80) at a dose of 2 mg/kg body weight or CID-diluent alone. Animals were monitored until death, euthanasia at predetermined time points, or euthanasia at first signs of distress (e.g., lethargy, huddled posture, inability to eat, ruffled fur, self-mutilation, vocalization, or >10% weight loss) or when tumor burden reached ~10% of body mass (2.5 g). At the time of sacrifice, final tumor volumes were calculated prior to tumor excision and fixation in 4% paraformaldehyde. Experimental protocols were reviewed and approved by the Animal Protocol Review Committee of Baylor College of Medicine. All mice were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care, and all animal studies were conducted in accordance with the principles and procedures outlined in Care and Use of Laboratory Animals (NIH) and in Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education (New York Academy of Sciences Ad hoc Committee on Animal Research).

Immunohistochemical Analysis. To determine apoptotic activity, 5-µm tumor tissue sections were stained by TUNEL technique (Trevisen, Gaithersburg, MD), according to the manufacturer’s instructions. Immunohistochemical staining for viral transduction and GFP transgene expression was performed using anti-GFP antibody (Boehringer Mannheim, Indianapolis, IN). Antigen retrieval was performed by applying 10 µM sodium citrate buffer (pH 6.0) and heating slides to 95°C for 5 min before incubation with anti-GFP antibody (1:100) at room temperature for 2 h. After three washes, slides were...
incubated with secondary antibody (1:400; Vector Laboratories, Burlingame, CA) for 1 h. Slides were counterstained with hematoxylin solution (Richard-Allan Medical, Richland, MI), dehydrated, and coverslipped.

The percentage of apoptotic TUNEL-positive cells and the area of GFP immunoreactivity were quantified using light microscopy by investigators blinded to treatment groups. Three nonoverlapping fields sampled from areas of highest staining were analyzed by counting the total number of stained and unstained cells. Multiple sections from each tumor were analyzed. Serial sections processed without incubation in primary antibody provided a control for specificity. Tumor specimens exhibiting high GFP and/or TUNEL activity were selected as positive controls for subsequent experiments.

**Statistical Analyses.** Differences in tumor volume, in relative increase of tumor volume, and in apoptotic activity were assessed using ANOVA. Multiple comparisons were conducted, when the overall test was significant (one-way ANOVA followed by Fisher’s least significant difference). The relative increase of tumor volume was defined as the percentage change in tumor volume between two consecutive measurements. Survival rates were calculated by the Kaplan-Meier method, and differences between the treatment groups were determined with the log-rank test. Statistical significance in this study was set as \( P < 0.05 \). All reported \( P \)s are two-sided. All analyses were performed with the SPSS statistical package (SPSS version 10.0 for Windows).

**RESULTS**

**CID-mediated Activation of Downstream Chimeric Caspases Triggers Apoptosis in a Broad Panel of Prostate Cancer Cell Lines.** Chemically induced dimerization of death domain-containing receptors, such as Fas, and downstream signaling intermediates, such as FADD and caspases, lead to their activation with ensuing cellular apoptosis. The inducible Fas was comprised of the cytoplasmic domain of Fas fused to two mutant FKBP12 moieties (F'), carrying the F36V mutation that permits high-affinity binding to third generation CID, such as AP20187 or AP1903 (20). This chimeric Fas molecule, MF2-Fas, was further targeted to the plasma membrane with the myristoylation (M)-targeting domain of c-Src, as described previously (21). All other CID-responsive molecules used in this study, including SFpk3-FADD (iFADD), SFpk3-Casp8 (iCasp-8), SFpk3-Casp1 (iCasp-1), and SF2-Casp3 (iCasp-3), are nontargeted, soluble (S) proteins and predominately reside in the cytoplasm as determined by immunofluorescence (19); iFADD contains only the NH2-terminal death effector domain of FADD (residues 1-125), whereas all iCasp3s contain one, or more, FKBP5s fused to the NH2-terminal prodomain of the nominal caspase. Furthermore, the choice of CID, either “third generation” AP1903/AP20187 or “second generation” FK1012/AP1510, was solely dependent on the choice of CID-binding domain, either F', or FKBP12-G89P/I90K (Fpk), respectively, and did not affect the relative inducibility of the constructs at the concentration of CID used for these studies (data not shown).

To determine the most broadly effective inducible death switch for treating prostatic neoplasms, we transiently transfected the panel of CID-inducible Fas signaling intermediates into a broad spectrum of androgen-responsive and -independent prostate cancer cell lines and compared their abilities to trigger apoptosis (Fig. 1). Each cell line was also transfected with the reporter plasmid, pGL2-Control, which constitutively expresses luciferase under the transcriptional control of the strong CMV promoter/enhancer. Numerous previous studies have shown that a decrease of reporter activity correlates strongly with an increase in apoptosis (21, 22). Twenty-four h after CID addition to each aliquot of transfected cells, cytoplasmic luciferase was assayed and compared with aliquots of diluent-treated cells. Although upstream signaling molecules Fas, FADD, and caspase-8 can moderately trigger apoptosis in some prostate cancer lines, such as TRAMP-C2 and LNCaP (data not shown), downstream caspase-1 and caspase-3 can trigger apoptosis in all cells tested to date, including androgen-independent lines such as PC-3, DU145, and Tsu-Prl. Thus, on the basis of their wide applicability, iCasp-1 and iCasp-3 were chosen for additional studies.

**CID-dependent Apoptosis of Prostate Cancer Cell Lines Treated with iCasps.** Because of the high transduction efficiency of adenovirus, iCasp-1 and iCasp-3 were subcloned into replication-defective adenovirus 5 vectors using the pAdEasy system (18). Because the pAdTrack shuttle vectors also incorporate GFP, transduction efficiency in various cell lines could be determined by assaying fluorescence. Prostate cancer cell lines, including LNCaP, PC-3, TRAMP-C2, TRAMP-C2G, and AT6 (data not shown), infected at increasing titers (MOI, 0–40) demonstrated varying transduction efficiencies, as determined by flow cytometry. Of the cell line lines tested, human lines, such as PC-3 and LNCaP (data not shown), were more efficiently transduced than murine lines (Fig. 2).

Ad-GiCasp1 and Ad-GiCasp3 were both effective in inducing apoptosis within 24 h in LNCaP, PC-3, TRAMP-C2, and TRAMP-C2G, and this effect was typically greatly augmented by exposure to CID (Fig. 3). After infection with Ad-GiCasp1 and CID treatment, the percentage of apoptotic PC-3 cells increased from 20% at an MOI of 2.5 to 94% at MOI 40. In contrast, in the absence of CID, no significant apoptosis was detected. LNCaP cells, however, displayed significant CID-independent apoptosis that was further augmented by CID addition. Two days after infection with Ad-GiCasp1 and 24 h after CID addition, the surviving fraction of cells in decreasing order of sensitivity was LNCaP (4%), PC-3 (6%), TRAMP-C2G (12%), and TRAMP-C2 (25%), correlating with the increased transduction of PC-3 and LNCaP cells versus TRAMP cell lines by
adenoviral 5-based vectors. Additionally, the rodent prostate cancer line, AT6 from the Dunning rat model, also displayed sensitivity and transduction rates similar to TRAMP-derived murine cells (data not shown). Similarly, Ad-G/iCasp3 infection plus CID also resulted in significant cell death compared with mock infection. However, Ad-G/iCasp1 was consistently more effective than Ad-G/iCasp3 in inducing apoptosis. Therefore, Ad-G/iCasp1 was selected to study the safety and efficacy of treating prostate-derived tumors in vivo.

Treatment of TRAMP-C2 s.c. Tumors with iCasps. In a pilot standard dose-escalation experiment, we determined the safety and efficacy of injecting Ad-G/iCasp1 (5 × 10⁹ to 2.5 × 10¹⁰ viral particles/25 µl) into s.c. TRAMP-C2 tumors (data not shown). Tumor-bearing animals were randomly assigned to receive either Ad-G/iCasp1 or control vector diluent. Each animal was further assigned to treatment with i.p. injections of either AP20187 or control CID-diluent on days 1 and 2 after adenoviral injection. Although there was only a marginal effect on tumor growth, we observed highly efficient interspersed areas of transgene expression in a viral titer-dependent manner. Transgene expression as measured by the extent of anti-GFP staining continued to increase at each time point examined. As expected, the highest viral dose (2.5 × 10¹⁰ viral particles) reflected the highest transduction efficiency, yet did not cause any overt host systemic toxicity.

In a subsequent experiment, we administered CID (AP20187) both 2 and 4 days after viral injection and examined the kinetics of GFP expression over an extended time line (Fig. 4A). Two tumors/group were harvested on days 2, 6, and 10, and the remaining four/group on day 18. Anti-GFP staining of tumor tissues confirmed that although viral transgene expression was undetectable on day 2 after viral

Fig. 3. In vitro susceptibility of prostate cancer cell lines to Ad-G/iCasp1 or Ad-G/iCasp3 at increasing MOI with and without CID (AP20187). The percentages of viable cells of human (PC-3 and LNCaP) and mouse (TRAMP-C2 and TRAMP-C2G) prostate cancer cell lines were measured by a crystal violet staining assay after infection with Ad-G/iCasp1 or Ad-G/iCasp3 with (○) or without (□) AP20187. Columns represent the percentage of surviving cells (means; bars, SE). Note: significant drug-independent induction of apoptosis was caused by Ad-G/iCasp1 or Ad-G/iCasp3 in LNCaP cells compared with more strictly CID-dependent apoptosis in the other virally transduced cell lines.
injection, marked expression was observed on day 6, which decreased thereafter (Fig. 5). TUNEL staining revealed extensive areas of apoptosis in tumors treated with Ad-G/iCasp1 plus CID (data not shown). Consistent with the kinetics of GFP expression, the quantity of apoptosis was minimal on days 2 and 3, increased by day 6, and peaked dramatically at day 10 in tumors treated with Ad-G/iCasp1 plus CID (data not shown). In contrast, control tumors treated with virus but not CID and those treated without virus demonstrated limited evidence of apoptosis (data not shown).

We next assessed the efficacy of Ad-G/iCasp1 plus CID treatment in a larger experiment (Fig. 4B), which included 12 mice/control group and 20 mice in the Ad-G/iCasp1 plus CID-treated group. s.c. tumors were injected with Ad-G/iCasp1 (2.5 × 10^{10} viral particles) or vector-diluent and treated with a single dose of i.p. CID or CID-diluent on day 6 after viral injection. Three to five tumors from each group were harvested randomly on days 10 and 16 after viral injection, whereas the remaining animals were sacrificed when tumor burden reached ~10% of body weight (2.5 g) or when animals appeared in distress by the criteria described in “Materials and Methods.”

Tumor volumes were measured every other day and compared between the three groups, beginning on the day of viral or vector-diluent tumor injection. Between days 4 and 6, animals treated with Ad-G/iCasp1 with or without CID experienced a slower increase in mean tumor volume compared with those treated with vector-diluent plus CID (P > 0.05), which may have reflected a transient inhibitory effect of the virus on tumor growth (Fig. 6). Between days 6 and 8, however, all tumors resumed growth at the same rate. Moreover, between days 8 and 10 (2 and 4 days after CID or CID-diluent administration, respectively), animals treated with Ad-G/iCasp1 plus CID experienced a significantly slower increase in tumor volume than those treated with vector-diluent plus CID (P < 0.001) or Ad-G/iCasp1 plus CID-diluent (P = 0.044), which were not significantly different from each other (P = 0.101). Reflecting these effects on tumor growth, no significant difference in tumor volume (P > 0.13) was noted between any of these groups from the first day of virus inoculation up to day 8. However at day 10, tumors treated with Ad-G/iCasp1 plus CID were significantly smaller than those treated with vector-diluent plus CID (mean, 164.7 versus 254.5 mm³; P = 0.003) or with Ad-G/iCasp1 plus CID-diluent (mean, 207.8 mm³; P = 0.005), which were also significantly different from each other (P = 0.017) on day 10 but not thereafter. Tumors treated with Ad-G/iCasp1 plus CID remained significantly smaller than those treated with Ad-G/iCasp1 plus CID-diluent through day 16 (P < 0.035) and also significantly smaller than those treated with vector-diluent plus CID through day 18 (P < 0.022). All groups had almost identical relative increases in tumor volumes after day 10 (P > 0.118), suggesting that effects on growth suppression occurred rapidly during the first few days after treatment up to day 10, but thereafter, all tumors grew at similar rates.

In addition to measurements of tumor size, TUNEL and anti-GFP staining was done on tumor sections from three to five total mice/group selected randomly and sacrificed on days 10 and 16 after viral injection. For each measurement, three nonoverlapping fields were

Fig. 5. Detection of viral transgene expression using anti-GFP in a TRAMP-C2 s.c. model. Animals were treated with 2.5 × 10^{10} Ad-G/iCasp1 plus CID-diluent and randomly sacrificed for tumor harvesting on days 2, 6, 10, and 18 after viral injection. Caspase-1 transgene expression was undetectable on day 2 (A), increased dramatically by day 6 (B), decreased by day 10 (C), and reached even lower levels by day 18 (D). Fields of highest staining are shown.
chosen from the areas displaying maximum apoptotic activity. Ad-G/iCasp1 plus CID-treated tumors showed a dramatic induction of apoptosis at day 10, with greater than 7-fold ($P < 0.001$) and 2.5-fold ($P < 0.001$) increases in the number of apoptotic cells compared with tumors not treated with virus and tumors treated with virus but not CID, respectively (Figs. 7 and 8). At day 16, the apoptotic activity of the tumors treated with Ad-G/iCasp1 plus CID and those treated with Ad-G/iCasp1 plus CID-diluent decreased by 48 and 43%, respectively, compared with day 10 ($P < 0.001$; Figs. 7 and 8), demonstrating the rapid kinetics by which these iCasps can initiate and complete apoptosis. The observed cytotoxicity of virus alone, both in vitro and in vivo by both tumor volume measurements and TUNEL assay, is consistent with the well documented ability of caspase overexpression to promote apoptosis (23–25). However, these experiments clearly demonstrate that this basal level of proapoptotic ability can be dramatically enhanced by purposeful caspase dimerization.

Areas of TUNEL immunoreactivity almost completely overlapped with areas staining positive for GFP, indicating that apoptosis occurred almost exclusively in cells expressing the viral transgenes (Fig. 8), clearly demonstrating the absence of a significant bystander effect. The distribution of transgene expression and the corresponding distribution of apoptotic bodies were spatially restricted to the surrounding zone of tissue along the injection tract of Ad-G/iCasp1 (Figs. 8 and Fig. 9). Fig. 9 shows a section from a tumor treated with Ad-G/iCasp1 plus CID harvested on day 10 and stained for apoptosis. The apoptotic bodies circumferentially surrounded the needle tract, and the extent of apoptosis measured perpendicularly to the needle tract was only 0.525 mm (diameter). Tumors treated with vector-diluent + AP20187 showed a transient decrease in tumor growth between days 4 and 6 in Ad-G/iCasp1-treated groups, which was further magnified in the Ad-G/iCasp1 plus CID-treated group beginning on day 8 (2 days after CID administration) and ending on day 10. Thereafter, tumor growth rates based on tumor volumes were almost identical, suggesting that growth suppression takes place rapidly in the first few days after treatment up to day 10 and thereafter diminishes. Bars, SD.

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**Fig. 6.** Tumor growth posttreatment in a TRAMP-C2 s.c. model. s.c. tumors, 12/control group and 20/Ad-G/iCasp1 plus CID-treated group, were directly injected with Ad-G/iCasp1 or vector-diluent and treated with a single dose of i.p. CID or CID-diluent on day 6 after viral injection. Three to five tumors from each group were harvested randomly on days 10 and 16 after viral injection. Tumor growth was similar in all groups until day 4. A transient decrease in tumor growth was observed between days 4 and 6 in Ad-G/iCasp1-treated groups, which was further magnified in the Ad-G/iCasp1 plus CID-treated group beginning on day 8 (2 days after CID administration) and ending on day 10. Thereafter, tumor growth rates based on tumor volumes were almost identical, suggesting that growth suppression takes place rapidly in the first few days after treatment up to day 10 and thereafter diminishes. Bars, SD.

**Fig. 7.** Apoptotic activity at day 10 and 16 after viral inoculation in a TRAMP-C2 s.c. model. Three to five animals were randomly sacrificed for tumor harvesting on days 10 (□) and 16 (□). At both time points, a dose of $2.5 \times 10^{10}$ Ad-G/iCasp1 + CID led to a significant increase of apoptotic activity ($P \leq 0.002$) in conjunction with a significant reduction in tumor volume compared with controls (vector-diluent + CID and Ad-G/iCasp1 + CID-diluent). The apoptotic activity was determined by the TUNEL technique. Similarly, Ad-G/iCasp1 + CID-diluent induced significantly greater apoptosis relative to virus-diluent + AP20187 at days 10 and 16 ($P < 0.001$ and $P = 0.031$, respectively). Bars, SD.
diluent plus CID exhibited minimal apoptotic activity distributed in a diffuse pattern consistent with endogenous apoptotic activity (Figs. 7 and 8).

Furthermore, we performed a Kaplan-Meier survival analysis on host mice in this final experiment by observing the course of nonsacrificed animals to a maximum of 45 days. Mice with tumor burdens >10% of body weight or those that appeared moribund were scored as dead of disease and sacrificed. Healthy-appearing mice with tumors randomly harvested on days 10 and 16 were censored on the day of tumor harvest. Although no significant difference in survival was seen between the three groups, control diluent plus CID-treated mice began dying of disease earliest, beginning at day 27. There was a trend toward increased survival in the two treatment groups. Twenty % of noncensored host mice treated with Ad-G/iCasp1 plus CID, but none from the other two groups, remained alive at the completion of the experiment on day 45.

**DISCUSSION**

Apoptosis is a fundamental biological process defined by morphological changes and DNA fragmentation that serves to maintain tissue homeostasis by eliminating injured or unwanted cells (26). A wealth of emerging evidence suggests that dysregulation of apoptosis plays a key role in both the pathogenesis and progression of a multitude of disorders, including cancer (27). Furthermore, because most conventional anticancer therapies have been shown to kill tumor cells by inducing apoptosis, resistance to apoptosis is now increasingly rec-
Prostate cancer is a paradigm of apoptosis resistance, leading to cancer progression (28). For patients with metastatic, progressive disease, androgen deprivation is the first line of therapy and induces rapid apoptosis and tumor regression in most patients. Inevitably however, all patients eventually develop hormone-refractory disease associated with a high mortality rate within the ensuing 12 months (29, 30). The mechanisms for the emergence of androgen-independent cancer remain unproven. Defects in the androgen receptor axis have been observed in some patients with androgen-independent cancer (31, 32). Additionally, evidence demonstrating a major role for anti-apoptotic mechanisms has become more compelling (33). McDonnell et al. (28) demonstrated that the antiapoptotic protein Bcl-2 was commonly up-regulated in progressive hormone-refractory prostate cancer. Subsequently, up-regulation of additional antiapoptotic genes, such as survivin (34), caveolin (35), and Akt (36, 37), and inactivation of proapoptotic genes, such as p53 (38) and PTEN (39), have been implicated in the progression to androgen independence. These molecules, although diverse, all intersect apoptotic-signaling cascades triggered by environmental proapoptotic stimuli. For example, expression of the Fas receptor, a member of the tumor necrosis family of receptors, is commonly observed in human prostate cancer and prostate cancer cell lines, yet sensitivity to Fas-mediated apoptosis is inversely correlated with metastatic potential in these cells (40). Overcoming this apparent gauntlet of antiapoptotic mechanisms has become an important new strategy for developing more effective therapies for both early and progressive prostate cancer.

Toward this goal, we have evaluated the efficacy of a novel therapy that bypasses potential upstream antiapoptotic molecules by directly activating downstream effector caspases and triggering apoptosis. This novel gene therapeutic approach uses CID technology to increase the activation of ectopically expressed caspases and other proapoptotic molecules, leading to target cell apoptosis. Previously, we genetically fused one or two FKBP12 domains, containing high-affinity binding sites for the lipid-permeable dimerizing agents (CID), with the target molecules, caspase-1 and caspase-3 (and others), conferring CID-dependent aggregation and thus activation of these chimeric proteins (22). Herein, we initially demonstrate that inducible proteins that are downstream in the Fas signaling cascade, such as caspase-1, caspase-3, and caspase-9 (data not shown for caspase-9), can trigger apoptosis in a wide panel of prostate cells, whereas upstream signaling molecules, such as Fas, FADD, and caspase-8, are effective in a more limited array of these cells, presumably because of the putative upstream antiapoptosis checkpoints. We also demonstrated the robustness of this system for both in vitro and in vivo applications with the use of replication-defective adenoviral vectors expressing caspase-1 (Ad-G/iCasp1) or caspase-3 (Ad-G/iCasp3). We found that both human and mouse prostate cancer cell lines were efficiently transduced and underwent rapid and widespread apoptosis in a viral- and drug-dependent fashion. Furthermore, Ad-G/iCasp1 was more potent than Ad-G/iCasp3 in triggering CID-dependent apoptosis in all cell lines tested. Moreover, in vivo, treatment with Ad-G/iCasp1 plus CID was associated with a transient growth-suppressive effect on s.c. established prostate tumors and induced focally extensive apoptosis, which resulted in a trend toward improved long-term host survival without overt evidence for systemic toxicity. The small sample size (six animals in each control group and 12 in the maximally treated group) may have limited the statistical power of our study for detecting an association between treatment and disease-specific survival.

Our studies demonstrated that although overexpression of caspases alone led to a transient but significant decrease in tumor growth and tumor volume, the addition of CID, which forces chimeric caspase aggregation and activation, led to a more dramatic induction of apoptosis and a larger and longer sustained reduction in tumor growth and tumor volume. These effects were initially observed 4 and 6 days after viral or viral-diluent injection, which coincided with the onset of significant transgene expression (Fig. 5 and data not shown). Meanwhile, the effect of CID was apparent beginning on day 8 (2 days after CID administration). These data suggest that some transduced cells ectopically expressed caspases at levels below the threshold for basal activation and apoptosis. CID addition expands the radius of death to include cells with lower levels of caspases that are insufficient to activate downstream signals. These results are consistent with independent reports that caspase-3 cannot induce apoptosis of tumor cells if overexpressed (23, 25). In contrast, enforced dimerization of caspase-3 can efficiently trigger apoptosis of most, if not all, cells (19, 22). Moreover, activation of caspase-1, caspase-3, caspase-7, and caspase-9 by CID can be achieved at expression levels and CID concentrations far below that needed to activate the relatively activation-resistant caspase-3 (19).
These studies also demonstrated the utility of the TRAMP-C2 s.c. tumor model system for studying novel antiprostate cancer therapies. TRAMP cell lines are syngeneic B6 tumors and afford the possibility to investigate the role of the immune system in potentiating a caspase-mediated killing mechanism that cannot be studied in xenograft systems with nude/severe combined immunodeficient mice. In the syngeneic C57BL/6 background, s.c.-injected TRAMP-C2 cells form solid tumors in animals over a 4–6-week period. Morphologically, these tumors are firm, because of a dense extracellular matrix formed by the tumor cells, and exhibit a relatively slow doubling time, both traits reflective of human prostate cancer. This tumor feature may have limited the distribution of injected vector around the injection sites (Fig. 9) and may have blunted the overall effect of treatment on tumor volume. Faster growing, more porous experimental tumors may have been more amenable to gene therapy strategies by allowing a wider distribution of injected vector, but the ensuing results may have yielded an overly optimistic picture regarding the potential utility of these strategies ultimately for the treatment of human prostate cancer.

Regulation of apoptosis also promises to be useful in a wide range of nonmalignant disease states caused by the accumulation of unwanted cells or tissue, such as benign prostatic hyperplasia or atherosclerosis. Many previous attempts to treat such disorders have relied on overexpression of proapoptotic genes or on gene-directed enzyme prodrug therapy [reviewed by Spencer (41)]. Among the proapoptotic genes that have been tried in vivo are Fas ligand (42), caspase-8 (24), Bax (43), Bak (44), and p53 (45). Although the antiproliferative effects of all endogenous proapoptotic molecules can be mitigated by naturally occurring antiapoptotic “rheostat” proteins, such as c-FLIP, Bcl-2, and IAPs, in general, the effects of upstream signaling molecules (e.g., Fas, FADD, and caspase-8) can be blocked at a greater multitude of signaling steps than downstream molecules. Therefore, downstream signaling molecules, such as caspase-1, caspase-3, caspase-7, or caspase-9 can bypass most of these road blocks and are likely to be more broadly useful.

In addition to overexpression of proapoptotic molecules, an even larger number of both preclinical and clinical studies involving gene-directed enzyme prodrug therapy are under way [reviewed by Spencer (41)]. Among these are clinical trials based on HSV-tk (46, 47) and E. coli cytosine deaminase (48), plus combinatorial approaches that incorporate standard chemotherapy and radiotherapy. HSV-tk relies on the incorporation of a dideoxynucleoside analogue, such as GCV, to terminate DNA replication, leading to apoptosis and necrosis (11). A therapeutic index exists because HSV-tk can phosphorylate GCV at least 1000-fold more effectively than endogenous tk. Furthermore, the toxic metabolites of GCV are often directly disseminated from HSV-tk-expressing cells to surrounding untransduced cells, resulting in the death not only of the recipient (HSV-tk+) tumor cells but also of nonrecipient (HSV-tk–) tumor cells. Moreover, the use of cytosine deaminase gene therapy can result in a more profound bystander effect. Cytosine deaminase can convert the relatively benign nucleotide precursor 5-fluorocytosine to the chemotherapeutic drug 5-fluorouracil, which can interfere with both DNA and RNA replication (49). Because the activated prodrug 5-fluorouracil is lipid permeable, the cytosine deaminase-based bystander extends beyond metabolically coupled cells. Although suicide genes, based on reduced systemic toxicity prodrugs, promise a safer alternative to conventional chemotherapeutic treatments that rely on fully active drugs, the toxicity of prodrugs has not been completely eliminated. Better-targeted strategies to destroy only transduced cells without affecting neighboring tissues may add a supplementary safety level by reducing potential local toxicity and increasing control and predictability of the intended effect.

Recent events in human gene therapy trials have renewed the emphasis on safety, above all, as primary criteria for the development of novel gene therapy strategies (50, 51). Although viral transfer of proapoptotic molecules has been shown to significantly reduce the size of transplanted tumors because of direct cytotoxicity and often bystander mechanisms, the unregulated nature of most of these vectors limits the ability to control cytotoxicity once protein expression commences. A safer alternative would rely on a multiparticle activation scheme in which a binary suicide gene or inducible proapoptotic molecule, such as the ones described here, is regulated by a tissue-specific promoter. Mechanical or molecular targeting of the viral or nonviral DNA vector and even the prodrug could provide a third or forth tier of safety. Although the expanded killing zone provided by a local bystander effect might be an advantage for some diseased states, the potential increased toxicity to nontargeted tissue may be undesirable. We found that caspase-mediated killing provides a minimal, if any, bystander effect, because cell killing only occurred in cells expressing the caspase transgene. However, the therapeutic gene was only introduced into cells surrounding the injection site, limiting the eradication of the tumor. Thus, the caspase-based gene therapy may ultimately prove safer than gene therapy that includes local bystander killing, once the current inefficiency of gene transfer is overcome. For example, viral vector delivery in a solid-state vehicle was shown to improve vector delivery and the subsequent therapeutic effect in an experimental immunotherapy protocol (52). Moreover, combining apoptosis-based therapies with adjuvant immunotherapies may bridge the therapeutic gap between treating both localized organ-confined and metastatic lesions.

Although adenovirus-mediated gene therapy using CID-regulated caspases produced initially promising but incomplete antitumor responses, the subsequent outgrowth of surviving tumor cells led to the death or euthanasia of most hosts. Although encouraging, these findings suggest a potentially limited role of caspase-based cell killing as a monotherapy at the present time. Advances in gene delivery systems, improvements in vector injection techniques, improvement in modulation of antitumor cellular immune responses, and evaluation of combinatorial strategies that include conventional therapies should be the focus of future efforts. Nevertheless, the data presented suggest that iCasp-based artificial death switches can be part of safe and effective prostate cancer gene therapy.

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