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Antitumor Activity of Ad-IU2, a Prostate-Specific Replication-Competent Adenovirus Encoding the Apoptosis Inducer, TRAIL

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Abstract

In this study, we investigated the preclinical utility and antitumor efficacy of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) delivered by Ad-IU2, a prostate-specific replication-competent adenovirus (PSRCA), against androgen-independent prostate cancer. Through transcriptional control of adenoviral early genes *E1a*, *E1b* and *E4*, as well as TRAIL by two bidirectional prostate-specific enhancing sequences (PSES), expression of TRAIL as well adenoviral replication was limited to prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA)-positive cells. Ad-IU2 induced 5-fold greater apoptosis selectively in PSA/PSMA-positive CWR22rv and C4-2 cells than an oncolytic adenoviral control. Furthermore, prolonged infection with Ad-IU2 reversed TRAIL resistance in LNCaP cells. Ad-IU2 exhibited superior killing efficiency in PSA/PSMA-positive prostate cancer cells at doses 5- to 8-fold lower than required by a PSRCA to produce a similar effect. This cytotoxic effect was not observed in non-prostatic cells, however. As an enhancement of its therapeutic efficacy, Ad-IU2 exerted a TRAIL-mediated bystander effect through direct cell-to-cell contact and soluble factors such as apoptotic bodies. *In vivo*, Ad-IU2 markedly suppressed the growth of subcutaneous androgen-independent CWR22rv xenografts compared to a PSRCA at six weeks post-treatment (3.1- *vs*. 17.1-fold growth of tumor). This study demonstrates the potential clinical utility of a PSRCA armed with an apoptosis-inducing ligand.

Keywords

TRAIL; PSES; Prostate Cancer; Adenovirus; Gene Therapy

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Introduction

In 2008, it was estimated that prostate cancer would account for the most new cancer diagnoses, aside from skin cancer, at 186,320 men in the United States and would be the second most common cause of cancer deaths at 28,660 men.1 The management of prostate cancer remains challenging given the fact that as many as 15% of men with prostate cancer-specific mortality may initially have low-risk prognostic factors;2 furthermore, up to 9% of patients will be diagnosed with metastatic disease at initial presentation.3 Current therapies for men presenting with localized prostate cancer include radical prostatectomy, external beam radiation therapy and brachytherapy; however, 25% of these men will experience local failure within ten years of treatment.4, 5 Given that prostate cancer is dependent on activated androgens for growth,6 adjuvant androgen deprivation therapy (ADT) is well established as standard of care for high risk prostate cancer to slow the growth and dissemination of undetectable residual cancer cells at local or distant sites. Nearly all advanced prostate cancers will become hormone refractory within 14 to 20 months of initiating ADT,7, 8 and secondary hormonal therapy agents merely provide palliation without survival benefit for this fatal disease phenotype. Results from phase III clinical studies have recently suggested a role for docetaxel in the treatment of androgen-independent prostate cancer, demonstrating a two month survival advantage in addition to palliation.9, 10 Unfortunately, dose-limiting toxicities associated with such therapies limit the amount of the drug that can be delivered to the tumor, allowing the cancer to survive and fail therapy. While there is a pressing need to develop better systemic therapies to treat or prevent disseminated disease, there also exists an urgent need to develop more effective local treatment modalities. Due to its ability to selectively target prostate cancer cells through the use of tissue-specific promoters and its widely demonstrated clinical safety profile, molecular therapy for androgen-independent prostate cancer is an attractive adjuvant to conventional therapies.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also known as Apo-2 ligand, is a member of the tumor necrosis factor (TNF) family. Originally discovered because of its similarity to Fas-ligand, TRAIL is a 32 kDa type II transmembrane protein, whose C-terminal extracellular domain (amino acids 114-281) is homologous to other members of the TNF family.11, 12 After binding of homotrimeric TRAIL to the death domain-containing receptors DR413 and DR5,14 the apoptotic signal is transduced via the adapter molecule, Fas-associated death domain (FADD), which recruits the initiator caspases to the death-inducing signaling complex (DISC).15 TRAIL has been shown to preferentially kill tumor cells over normal cells, which reflects its role as a key tumor immunosurveillance molecule in the body.16 This marked specificity for cancer cells gives TRAIL a distinct advantage over other cancer therapies.

TRAIL expression has been detected in several normal human tissues, suggesting that TRAIL is not toxic to those cells *in vivo*.13 It is hypothesized that these cells are shielded from TRAIL by the surface expression of antagonistic decoy receptors. Three non-apoptotic signaling receptors exist for TRAIL, and these include DcR1 which lacks an intracellular death domain,14 DcR2 which contains a truncated death domain that activates anti-apoptotic NFκB signaling,17 and osteoprotegrin, a secreted receptor for TRAIL which also inhibits

osteoclastogenesis.18 Several prostate cancer cell lines including ALVA-31, DU-145 and PC-3 are extremely sensitive to TRAIL and undergo apoptosis when exposed; however, other cell lines such as LNCaP are highly resistant.19 Such mechanisms of resistance in cancer cells include overexpression of decoy TRAIL receptors, anti-apoptotic BCL-2 family members, and other inhibitor of apoptosis proteins (IAP).20 This resistance has been shown to be reversed by infection with adenovirus,21 treatment with chemotherapeutic agents such as paclitaxel, vincristine, etoposide, doxorubicin or camptothecin,22 or treatment of the cells with radiation therapy.23

Recent preclinical and clinical studies of molecular therapy approaches with TRAIL have involved a soluble form of the protein that is secreted out of producer cells and into the surrounding tumor matrix. Although repeated administration of soluble TRAIL was not toxic to normal tissues in mice24 and non-human primates,25 recent data suggest that cultured human hepatocytes may be sensitive to soluble forms of TRAIL.26, 27 To enhance the safety and clinical feasibility of this gene therapy strategy for high risk prostate cancer, we armed Ad-IU2, a prostate-specific replication-competent adenovirus (PSRCA), with full-length membrane-bound TRAIL under the transcriptional control of the chimeric prostate-specific enhancing sequence (PSES). Furthermore, to limit the replication of Ad-IU2 to prostate cancer cells, adenoviral *E1a*, *E1b* and *E4* genes were placed under control of PSES. PSES is comprised of the minimal sequences from prostate-specific antigen (PSA) androgen-responsive element core (AREc) and prostate-specific membrane antigen (PSMA) enhancer (PSME) that retained the highest prostate-specific activity. PSES was found to be active in PSA/PSMA-positive cells and demonstrated 5-fold higher activity than Rous sarcoma virus (RSV) promoter and activity equal to cytomegalovirus (CMV) promoter.28 As PSES is active only in PSA/PSMA-positive cells, adenoviral replication and TRAIL expression is limited to PSA/PSMA-positive cells. To date, this is the first prostate-specific promoter-driven TRAIL molecular therapeutic strategy for advanced prostate cancer.

Materials and Methods

Cell Culture

The packaging cell line HER911E4 stably expresses the adenoviral *E4* gene under control of the inducible *tetR* promoter29 and was derived from the human embryonic retinoblast (HER911) cell line which was transformed with a plasmid containing the adenoviral genome (bp 79-5789).30 HER911E4 cells were cultured in DMEM supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 1% penicillin-streptomycin (Gibco, Grand Island, NY), 0.1 mg/ml hygromycin B (Calbiochem, San Diego, CA) and 2 μg/ml doxycycline (Sigma, St. Louis, MO). To induce adenoviral *E4* gene expression, HER911E4 cells were cultured in medium without doxycycline for 24 hours prior to infection. CWR22rv is an androgen-independent, PSA/PSMA-positive prostate cancer cell line derived by the propagation of the androgen-dependent parental xenograft, CWR22, in nude mice.31 LNCaP is an androgen-dependent, PSA/PSMA-positive prostate cancer cell line established from a lymph node of a patient with metastatic disease.32 C4-2, an androgen-independent, PSA/PSMA-positive prostate cancer line, was derived by co-injection of LNCaP and bone stromal cells into nude mice.33 PC-3 is an androgen-independent, PSA/PSMA-negative

prostate cancer cell line that was originally derived from the bone marrow aspirates of a patient with bone metastases.34 DU-145, an androgen-independent prostate cancer cell, is PSA/PSMA-negative and was derived from a brain lesion from a patient with confirmed metastatic disease.35 All prostate cancer cell lines were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin. Adult human dermal fibroblasts (HDFa) were cultured in Medium 106 supplemented with 2% FBS, 1 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor and 10 μg/ml heparin (Cascade Biologics, Portland, OR). All cells were maintained in a humidified incubator at 37°C and 5% CO_2.

Adenoviral Vectors

Ad-IU2 was developed by modifying Ad-E4PSESE1a, the previously described PSRCA with a CMV promoter-driven enhanced green fluorescent protein (EGFP) marker.36 To construct Ad-IU2, human full-length TRAIL cDNA from pORF-hTRAIL (InvivoGen, San Diego, CA) was cloned downstream of PSES into pAd1020SfidA (OD 260, Boise, ID), the adenoviral cloning vector containing the left ITR and packaging signal, to make pAd1020SfidA-PSESTRAIL, which was further digested with *SfiI* to release the left ITR and PSES-TRAIL expression cassette. This fragment was cloned into pAd288E1b-E4PSESE1a,36 the modified adenoviral genome vector, and the ligation product was transformed into TOP10 *E. coli* competent cells (Invitrogen, Carlsbad, CA). The adenoviral genome (Fig. 1A) was released by digestion with *PacI* and transfected into HER911E4 cells with Lipofectamine 2000 (Invitrogen). Ad-IU2 was further amplified in HER911E4 cells and purified by CsCl centrifugation gradient and dialyzed, as described previously.36

Replication-competent control viruses used in this study include Ad-E4PSESE1a and Ad-IU1. Ad-IU1 was constructed in a similar fashion as Ad-IU2; however, a PSES-HSV-TK expression cassette replaces the PSES-TRAIL expression cassette. Without administration of a nucleoside analog prodrug, the only cytotoxicity provided by Ad-IU1 is due to replication. As a replication-defective control, Ad-ΔTATA-E1a, in which the *E1a* TATA box was deleted from the Ad-E4PSESE1a viral backbone, was used. To achieve equal bioactivity of Ad-IU2 and control viruses, a titer assay was performed. 1×10^4 HER911E4 cells were plated overnight in 96-well plates and infected with serial dilutions of Ad-IU2, Ad-IU1, Ad-E4PSESE1a or Ad-ΔTATA-E1a, so that 10 wells in every row received the same dose, ranging from 10^{-3} to 10^{-10}. Media were changed 24 hours after infection, and cells were observed daily under light microscopy for cytopathic effect. 7 days after infection, lethal dose (LD_{50}) was determined to be the dose at which 50% of cells or greater were killed (at least 5 wells per row). Viral titers were calculated as LD_{50} units (LDU) per μl. Conversions from viral particles (vp) to LDU were as follows: Ad-IU2, 1×10^{-5} LDU/vp; Ad-IU1, 6×10^{-6} LDU/vp; Ad-E4PSESE1a, 7.8×10^{-5} LDU/vp; and Ad-ΔTATA-E1a, 1.5×10^{-5} LDU/vp.

Western Blot Analysis

For TRAIL expression, 1×10^6 CWR22rv cells were cultured overnight in 6-well plates and infected with 0.01 LDU/cell Ad-IU2 or Ad-ΔTATA-E1a. As a positive control, CWR22rv cells were transfected with pORF-hTRAIL using Lipofectamine 2000. Media were changed

24 or 3 hours after infection or transfection, respectively. 48 hours after infection, cells were washed with cold PBS and harvested with radioimmunoprecipitation assay (RIPA) buffer containing 1 ml modified RIPA buffer, 20 μl 57 mmol/L phenylmethylsulfonyl fluoride and 2.5 μl phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Cell lysates were kept on ice for 1 hour, centrifuged to pellet debris, and supernatants kept at -70°C. To determine whether cleaved TRAIL was present in Ad-IU2 conditioned media, 1×10^6 CWR22rv cells were infected with 0.01 LDU/cell, as above. 48 hours after infection, the medium was harvested, centrifuged at 800 × g for 10 minutes, and the cell lysate was prepared as above. As positive controls, various dilutions from 100 to 25 ng/ml recombinant human TRAIL (rhTRAIL) (BioSource, Camarillo, CA) were loaded. Protein concentration was analyzed by Bradford assay (Bio-Rad, Hercules, CA), and 20 μg of protein (with 0.3 M DTT) were separated by 4-10% SDS-PAGE or 4-12% SDS-PAGE for the cleaved TRAIL experiment and transferred to a nitrocellulose membrane. Membranes were blocked overnight at 4°C in 5% fat-free milk and TBST and incubated with anti-human TRAIL primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were incubated with appropriate HRP-conjugated secondary antibodies, and specific binding was detected by ECL (Pierce, Rockford, IL).

Measurement of TRAIL Surface Expression

1×10^5 CWR22rv cells were plated overnight in 24-well plates and infected with 0.01 LDU/cell Ad-IU2 or Ad-IU1 or treated with PBS. Fresh media were replaced 24 hours after infection. 48 hours post-infection, cells were harvested and resuspended in 100 μl ice cold staining buffer (PBS containing 10% FBS and 1% sodium azide) in fluorescence-activated cell sorting (FACS) tubes. Cells were stained with PE-conjugated anti-human TRAIL antibody (Abcam, Cambridge, MA) for 1 hour at 4°C, protected from light. Cells were washed twice with ice cold PBS and resuspended in 500 μl staining buffer. Analysis was performed on a FacScan flow cytometer (BD Biosciences, Franklin Lake, NJ) and data analyzed using WinMDI 2.8 software.

Viral Replication Assay

1×10^6 CWR22rv, C4-2, LNCaP, PC-3 and DU-145 prostate cancer cell lines were seeded in 6-well plates overnight and infected with standardized doses of virus based on each cell line's infectivity.36 Media were changed 24 hours after infection, and cells were observed daily by light microscopy for cytopathic effect. Viral supernatants were harvested 3 days after infection by subjecting the cells and media to three freeze-thaw cycles and centrifuging to remove the cell debris pellet. HER911E4 cells were plated in 96-well plates and infected with serial dilutions of viral supernatant ranging from 1 to 10^{-11}, so that every well in each column received the same dose. Cells were examined for cytopathic effect on day 7, and LD_{50} was recorded as the dose causing cytopathic effect in at least four of eight wells in one column.

Measurement of Apoptosis Induction

CWR22rv, C4-2, LNCaP, PC-3 and DU-145 prostate cancer cells were seeded in 24-well plates overnight and infected with 0.01 LDU/cell Ad-IU2 or Ad-IU1 or treated with PBS. 24 hours after infection, media and cells were harvested, washed with PBS and resuspended in

100 μl binding buffer. LNCaP cells were also infected with 0.01 LDU/cell Ad-IU2 or Ad-IU1 or treated with PBS. 48 hours after treatment, media and cells were harvested, washed with PBS and resuspended in 100 μl binding buffer. Cells were stained with 2.5 μl each of Annexin V-FITC and propidium iodide (PI) (BD Biosciences, Pharmingen) for 15 minutes at room temperature and analyzed by FACS analysis as above. Cells that were single-positive for Annexin V-FITC or double-positive for Annexin V-FITC and PI were considered as positive for apoptosis.

In Vitro Cell Killing Assay

CWR22rv, C4-2, LNCaP and HDFa cells were seeded in 24-well plates overnight and treated with various doses of Ad-IU2, Ad-E4PSESE1a, Ad-ΔTATA-E1a or PBS. Media were replaced with fresh medium 24 hours after infection, and cells were maintained in culture, changing media every other day, until a cytopathic effect was observed under light microscopy. Once a cytopathic effect was evident, cells were fixed with 1% paraformaldehyde, washed twice with cold PBS, stained with 0.5% crystal violet solution for 10 minutes and washed with cold tap water. To quantitate the remaining attached cells, stained cells were permeabilized with 1% SDS and analyzed for optical density at 570 nm on a Spectra Max Plus spectrophotometer (Molecular Devices, Sunnyvale, CA). Cell viability was determined as the ratio of the A_{570} value for Ad-IU2-, Ad-E4PSESE1a- or Ad-ΔTATA-E1a-treated cells to the A_{570} value for untreated (PBS) cells at each viral dose.

Evaluation of Bystander Effect

CWR22rv cells were plated in a 12-well plate overnight and infected with 0.01 LDU/cell Ad-IU2 or Ad-IU1 or treated with PBS. 24 hours post-infection, cells were washed three times with cold PBS to remove residual virus, and fresh medium was replaced. PC-3 cells stably expressing a fusion of humanized *Renilla* luciferase (hrl) and monomeric red fluorescent protein (mrfp) reporter genes were co-cultured with the CWR22rv cells at a ratio of 3 CWR22rv cells to 1 PC-3 cell. The plasmid conferring expression, pcDNA3.1-CMV-hrl-mRFP, was constructed from pcDNA3.1-CMV-hrl-mrfp-ttk (a gift from Dr. Sanjiv Gambhir, Stanford University, CA) by deleting truncated thymidine kinase (ttk) from the vector. 24 hours after co-culture, media and cells were harvested, washed with PBS and resuspended in 100 μl binding buffer. Cells were stained with 2.5 μl Annexin V-FITC for 15 minutes at room temperature and analyzed by FACS analysis as above. Percent apoptotic PC-3 cells was determined as the fraction of Annexin V-FITC-positive cells in the mRFP-positive population.

To determine whether direct cell-to-cell contact was required to mediate a bystander effect, CWR22rv cells were seeded overnight in 6-well plates and infected with 0.01 LDU/cell Ad-IU2, Ad-IU1 or Ad-E4PSESE1a. 48 hours after infection, media were harvested, centrifuged at 800g for 10 minutes to remove dead cells and debris, and heat-inactivated at 56°C for 30 minutes. CWR22rv or PC-3 cells were seeded in 24-well plates overnight and treated with the heat-inactivated medium for 24 hours, at a ratio of 1:1. Media and cells were harvested and analyzed for apoptosis by FACS analysis as described above. To determine whether adenovirus was inactivated by heat-treatment, CWR22rv cells were seeded onto 24-well plates overnight and treated with conditioned medium from Ad-E4PSESE1a-infected

CWR22rv cells, as above, before or after heat-inactivation at 56°C for 30 minutes. 24 hours after treatment, cells were washed with cold PBS, harvested, fixed with 2% paraformaldehyde, resuspended in PBS, and analyzed for GFP expression by FACS analysis.

In Vivo Evaluation of Ad-IU2 Anti-Tumor Effect

CWR22rv xenografts were established by injecting 2×10^6 CWR22rv cells subcutaneously (SQ) into the flanks of 6 week-old male athymic nude mice. One week after injection, mice were anesthetized and bilateral orchiectomies were performed to ablate the production of androgens. Once tumors were established (33.5 to 65.45 mm^3), mice were injected intratumorally with 2×10^4 LDU Ad-IU2, Ad-IU1 (PSRCA control) or PBS (vehicle control). Tumor sizes were monitored weekly, and tumor volumes were calculated as $(L^2 \times W) / (\pi/6)$. Tumor data was presented as fold-increase in tumor size relative to initial size at time of treatment. Mice were sacrificed at 6 weeks, and tumors were harvested, fixed in formalin and embedded in paraffin. All animal procedures were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee (IACUC). Tumor sections were deparaffinized with xylene, hydrated in ethanol and distilled water, and stained with Hematoxylin and Eosin (H&E). Tumor sections were evaluated for *in situ* apoptosis using a fluorometric terminal dUTP nick-end labeling (TUNEL) assay (Promega, Madison, WI). Nuclei were counterstained with DAPI and tumor sections visualized by confocal microscopy on a Bio-Rad MRC1024 laser scanning dual-photon confocal microscope (Bio-Rad).

Statistical Analysis

Statistical significance was determined using two-way ANOVA with Bonferroni's post-test or unpaired *t* test, as indicated, using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). Statistical significance was defined as a P value < 0.05, or better. Experiments were plated in triplicate, unless otherwise noted, and performed as at least three independent experiments.

Results

Ad-IU2 Delivered Surface-Bound TRAIL and Replicated Effectively in PSA/PSMA-Positive Cells

The structure of Ad-IU2 (Figure 1A) is based on the PSRCA, Ad-E4PSESE1a, in which the *E1* promoter was deleted and *E1a* moved to the right ITR *E4* region under control of the bidirectional PSES enhancer sequence.36 Full-length, membrane-bound TRAIL cDNA was inserted at the left ITR in the *E1a* region upstream from adenoviral *E1b*, both under the control of PSES. As depicted in Figure 1B, full-length TRAIL protein expression was confirmed by western blot in PSA/PSMA-positive CWR22rv prostate cancer cells. Immunoblot of Ad-IU2-infected cells revealed a 32 kD band comparable in size to that of full-length TRAIL expressed in pORF-hTRAIL-transfected cells. Infection with Ad-ΔTATA-E1a confirmed the lack of endogenous TRAIL expression or the upregulation of TRAIL by adenoviral infection in CWR22rv cells. To confirm that TRAIL was expressed on the cellular membrane of infected cells, we performed FACS analysis for cell-surface

expression of TRAIL. Infection of CWR22rv prostate cancer cells with Ad-IU2 resulted in a significant enhancement of TRAIL surface expression (25.97%, 95% CI 20.41 to 31.54%, p <0.001), compared to infection with Ad-IU1 (Figure 1C).

Previous studies have demonstrated that adenoviral vectors expressing apoptosis-inducing transgenes and death ligands replicate poorly due to decreased adenoviral gene expression and producer cell toxicity. This results in disabling replication efficiencies, low production yields and poor transduction efficiencies.37 Expression of TRAIL protein in cells may be inversely proportional to the ability of the virus to replicate in those cells; therefore, we performed a replication assay in prostate cancer cell lines to determine whether Ad-IU2 replication efficiency was inhibited by TRAIL expression. Ad-IU2 replicated as efficiently as the PSRCA, Ad-E4PSESE1a, in PSA/PSMA-positive cells; however, Ad-IU2 failed to propagate in PSA/PSMA-negative cells, resulting in viral output yields comparable to that of the replication-deficient virus, Ad-ΔTATA-E1a (Table 1). These results demonstrate that Ad-IU2 replication is not hindered by the expression of TRAIL in PSA/PSMA-positive prostate cancer cells. In addition, these data demonstrate the selectivity of Ad-IU2 replication for PSA/PSMA-positive prostate cancer cells, in which expression of adenoviral early genes is controlled by PSES.

Ad-IU2 Effectively Induced Apoptosis and Reversed Resistance in PSA/PSMA-Positive Prostate Cancer Cells

Apoptosis-inducing agents such as TRAIL have shown promising clinical potential against solid tumors;21, 38, 39 therefore, we tested the ability of Ad-IU2 to induce apoptosis in prostate cancer cell lines. PSA/PSMA-positive prostate cancer cells, CWR22rv, C4-2 and LNCaP, as well as PSA/PSMA-negative cell lines, PC-3 and DU-145 were treated with PBS, Ad-IU2 or Ad-IU1 for 24 hours and analyzed for apoptosis by FACS analysis. As depicted in Figure 2A, apoptosis induction within 24 hours of Ad-IU2 infection in CWR22rv and C4-2 cells was nearly 5-fold higher than baseline or Ad-IU1-induced levels. As expected, no apoptosis above baseline was detected in PSA/PSMA-negative PC-3 and DU-145 prostate cancer cell lines. Likewise, no apoptosis was detected in the PSA/PSMA-positive LNCaP cell line, which has been shown to be highly resistant to TRAIL-mediated apoptosis due to high AKT activity.19 This TRAIL resistance was overcome however, by prolonged infection with Ad-IU2. LNCaP cells infected with Ad-IU2 for 48 hours demonstrated greater than 5-fold induction of apoptosis above that of control virus infection (Figure 2B). This is consistent with previous reports demonstrating that TRAIL resistance can be overcome by co-expression of TRAIL and adenoviral E1a. 40

Ad-IU2 Effectively Killed Prostate Cancer Cells, While It Spared Normal Cells

To assure that apoptosis induction and viral replication within cells was sufficient to kill prostate cancer cells, an *in vitro* killing assay was performed on CWR22rv, C4-2, LNCaP and adult human dermal fibroblast (HDFa) cells. Following treatment with PBS or serial dilutions of Ad-IU2, Ad-E4PSESE1a or Ad-ΔTATA-E1a, cytopathic effect of the virus was monitored by light microscopy and viral killing was determined by staining attached cells with crystal violet. The oncolytic effect was observed earliest in C4-2 and LNCaP cells, resulting in assay endpoints of 3 days post-infection for C4-2 and LNCaP and 4 days post-

infection for CWR22rv. This difference in time course between prostate cancer cell lines is consistent with delayed replication and transgene expression due to slightly reduced PSES transcriptional activity in CWR22rv cells.28 Ad-IU2 exhibited greater killing efficiency in PSA/PSMA-positive prostate cancer cells at doses markedly lower than the PSRCA, Ad-E4PSESE1a. As depicted in Figure 3A-C, the dose of Ad-IU2 required to kill at least 50% of CWR22rv, C4-2 and LNCaP cells was 5-, 6- and 8-fold lower than that of Ad-E4PSESE1a, respectively. Of note, in our hands, LNCaP cells are extremely sensitive to viral infection, as depicted in Figure 3C, and although Ad-ΔTATA-E1a is a replication-deficient adenovirus, it still induces cytotoxicity due to an intact *E3* region. To demonstrate specificity for PSA/PSMA-positive cells, this experiment was repeated using normal human fibroblasts. Despite treatment of cells for a longer period of time, Ad-IU2 produced no cytotoxicity in HDFa cells (Figure 3D). Cytotoxicity in normal prostate epithelial cells was not tested, as Ad-IU2 would be expected to replicate in these PSA/PSMA-positive cells; however, this does not pose a safety concern because the prostate is a non-vital organ in the post-reproductive male. These data suggest that expressing TRAIL in a PSRCA enhanced its anti-tumor cytotoxicity, while maintaining its PSA/PSMA-specificity.

Ad-IU2 Demonstrated a Bystander Effect in PSA/PSMA-Negative Prostate Cancer Cells

Due to limited viral transduction efficiency *in vivo* and the heterogeneity of human prostate tumors with regards to PSA/PSMA-expression, the ability to target and destroy prostate cancer cells in which a PSRCA cannot replicate and lyse the cell is critical to prevent the development of foci of untreated cells within a tumor. The killing power of Ad-IU2 could be enhanced through cell-to-cell contact of neighboring cells with infected prostate cancer cells or cell contact with the apoptotic bodies from dying cells. To determine whether Ad-IU2 imparted a bystander killing effect on neighboring PSA/PSMA-negative prostate cancer cells, we co-cultured Ad-IU1- or Ad-IU2-infected CWR22rv cells with mRFP-stably transfected PC-3 cells and detected the level of apoptosis induction in the mRFP-labeled PC-3 cells. As depicted in Figure 4A, PC-3 cells, which failed to undergo apoptosis induction following direct infection by Ad-IU2, due to a lack of PSA and PSMA expression (Figure 2A), exhibited a 4-fold induction of apoptosis above the level induced by Ad-IU1 co-culture when co-cultured with Ad-IU2-infected CWR22rv cells.

To determine whether direct cell-to-cell contact was necessary to produce a bystander killing effect, we tested the ability of conditioned media from Ad-IU2-infected cells to elicit a similar response. Conditioned media collected from CWR22rv cells infected with Ad-IU2 or control virus were heat-treated to inactivate any adenoviral particles present. When treated with conditioned media, both CWR22rv and PC-3 cells achieved a similar level of apoptosis induction (Figure 4B). To assure that this effect was not directly mediated by adenoviral infection and that adenovirus was inactivated effectively by heat treatment, CWR22rv cells were treated with conditioned media from Ad-E4PSESE1a-infected CWR22rv cells. Heat inactivation of the conditioned media resulted in a 40-fold reduction in GFP-positivity compared to conditioned media without heat inactivation (Figure 4C), demonstrating effective inactivation of adenovirus by heat treatment of the conditioned media. These results suggest that unidentified soluble factors can mediate the Ad-IU2 bystander effect. This in turn would enhance distribution of the cytotoxic effects throughout

the entire tumor, as direct cell-to-cell contact is not required. To determine whether soluble TRAIL was cleaved from the cell membranes of infected cells and contributed to the bystander killing of prostate cancer cells, we performed western blot analysis on conditioned media from Ad-IU2-infected CWR22rv cells for TRAIL protein. As shown in Figure 4D, cell lysate from Ad-IU2-infected CWR22rv cells expressed full-length, 32 kD TRAIL protein; however, no band was detected in the conditioned media from these cells. As a control, various dilutions of rhTRAIL were immunoblotted from 100 to 25 ng/ml, and the intensity of the 18 kD bands decreased with respect to protein concentration. These results confirm that soluble TRAIL was not secreted or cleaved from Ad-IU2 infected cells at concentrations suitable to induce cytotoxicity in prostate cancer cells.

Ad-IU2 Inhibited the Growth of Subcutaneous Androgen-Independent CWR22rv Xenografts

Previously, we investigated the oncolytic potential of Ad-E4PSESE1a, a PSRCA, which significantly inhibited the growth of CWR22rv xenografts as compared to control virus; however, the response only lasted two weeks, after which the tumor growth exceeded the rate of oncolysis. Rapid intratumoral viral replication and spread peaked at 3 days and was diminished by 1 week after injection.36 For this reason, we determined whether TRAIL could augment the *in vivo* antitumor effects of a PSRCA. Androgen-independent CWR22rv human prostate cancer xenografts were established SQ in the flanks of castrated athymic male mice and injected with Ad-IU2, Ad-IU1 (replication-competent control) and PBS (vehicle control). Ad-IU2 significantly suppressed the growth of CWR22rv tumor xenografts as compared to Ad-IU1 (3.1-*vs*. 17.1-fold growth of tumor, respectively). 4 weeks after treatment, Ad-IU1-treated tumors began to fail therapy, resulting in a rebound of tumor growth. On the other hand, Ad-IU2 continued to inhibit tumor growth through the 6-week end-point of the study. Mock-treated mice were sacrificed at 5 weeks due to overwhelming tumor burden (Figure 5A). Of the nine tumors treated with Ad-IU2, six responded favorably with partial regression in four of six or complete regression in two of six tumors. Of the three tumors that failed, two were significantly suppressed compared to Ad-IU1 tumors at 6-weeks (Figure 5B). Given the fact that CWR22rv xenografts are clonogenic, the variation in treatment outcome may be attributed to incomplete tumor infiltration or leakage of virus at the time of injection. Histological examination of PBS-treated tumors revealed healthy cells arranged in normal tumor architecture with significant tumor vasculature in the margins of the growing tumor (Figure 5C). Ad-IU1-treated tumors were characterized by scattered necrotic patches surrounded by healthy tumor cells, indicative of incomplete oncolysis due to limited viral replication and propagation throughout the entire tumor mass (Figure 5D). Although patches of healthy tumor cells remained within the Ad-IU2-treated tumors, necrotic centers of viral replication and oncolysis were more diffuse throughout the entire tumor. Furthermore, cells immediately surrounding the necrotic centers appeared unhealthy with condensed nuclei, indicating spread of the cytotoxic and apoptotic effect beyond the necrotic centers (Figure 5E). To determine whether apoptosis contributed significantly to the tumor killing process, *in situ* TUNEL assays were performed on the tumor sections. No apoptotic nuclei were detected in the control tumors (Figure 5F-G). On the other hand, Ad-IU2-treated tumors displayed marked apoptosis in the margins surrounding necrotic centers of oncolysis (Figure 5H).

These data suggest that TRAIL potentiated the *in vivo* killing power of a PSRCA through apoptosis induction in cells beyond the margin of viral replication.

Discussion

Early gene therapy clinical trials for cancer involved replication-deficient adenoviral vectors due to safety concerns of nonspecific viral replication in immunocompromised patients; however, these studies were limited by poor viral transduction efficiencies. To overcome this, replication-competent oncolytic adenoviral vectors were developed to kill cancer cells directly and further propagate the vector. The first tumor-specific oncolytic adenovirus developed was ONYX-015, in which *E1b-55kd* was deleted to restrict replication to p53-deficient cancer cells;41 however, later studies have demonstrated replication independent of p53 status.42 ONYX-015 has been widely tested in clinical trials and has demonstrated inefficient cell lysis and viral replication, resulting in poor clinical outcomes.43, 44 In a second approach, the adenoviral immediate early *E1a* gene was placed under control of tissue-specific promoters.45-47 To achieve greater control of viral replication, both *E1a* and *E1b* genes were placed under control of multiple or single bidirectional promoters.48, 49 We developed a prostate-specific oncolytic adenovirus, called Ad-E4PSESE1a, in which adenoviral *E1a* and *E4* genes were controlled by the bidirectional PSES enhancer.36 Previous studies have demonstrated that TRAIL-mediated apoptosis potentiated oncolysis and spread of a replication-competent adenovirus throughout a tumor.50 Therefore, to enhance the oncolytic and therapeutic potential of this PSRCA, we incorporated TRAIL cDNA under the transcriptional control of PSES, making Ad-IU2 the first prostate-specific TRAIL vector.

Currently, four strategies have been developed to deliver TRAIL via conditionally-replicating adenoviral vectors. Ad5/35.IR-E1a/TRAIL is an adenovirus containing an inverted *E1a* sequence that replicated exclusively in tumor cells via complementation with unknown cellular factors.51 Through an adenoviral replication-dependent homologous recombination event, the bicistronic 3′ to 5′ TRAIL-IRES-E1a cassette is flipped to the correct orientation and expressed under control of the universal RSV promoter.39 In a second tumor-specific approach, a CMV-TRAIL expression cassette was incorporated into the *E1b-55kd*-deleted adenovirus, ONYX015. To improve the anti-tumor efficacy of this virus, ZD55-hTRAIL, against colorectal carcinoma, it was administered in combination with 5-FU.52 Ad/TRAIL-E1 contains two duplicated synthetic promoters in close proximity comprised of the full hTERT promoter and minimal sequences from the CMV promoter to control the expression of both *E1a* and TRAIL as separate transcripts.53 The final approach achieved tissue-specific replication and TRAIL expression for hepatocellular carcinoma using the α-fetoprotein promoter to control the expression of a bicistronic *E1a*-TRAIL cassette.54 Our strategy is unique to the previously described replication-competent TRAIL vectors in that tight tissue-specific regulation of adenoviral replication was achieved by controlling three early adenoviral genes, *E1a*, *E1b* and *E4*. Furthermore, in our study TRAIL was co-expressed with adenoviral *E1a* in PSA/PSMA-positive cells without the use of bicistronic elements, which can result in decreased gene expression of transgenes downstream of the internal ribosomal entry sequence (IRES), or universal promoters, which may induce adenoviral replication outside of the target tissue. The use of a prostate-specific

promoter such as PSES to control viral replication and transgene expression enhanced the safety of this vector, while enabling its use for both locally advanced and metastatic prostate cancer.

We hypothesized that arming a prostate-specific oncolytic adenoviral vector with TRAIL would enhance the anti-tumor efficacy of a replication-competent adenovirus. In this approach, prostate cancer cells would undergo apoptosis by the tumor-specific apoptosis inducer, TRAIL. Furthermore, the replication of the adenoviral vector would kill prostate cancer cells directly by oncolysis and result in amplification of both adenoviral vector and viral transduction efficiency. Finally, through a TRAIL-mediated bystander effect, cytotoxicity of this molecular therapy would spread throughout the tumor, killing nontransduced cells.

We have demonstrated that while TRAIL was expressed in PSA/PSMA-positive prostate cancer cells, this had no mal-effects on viral replication (Table 1). This is consistent with our ability to produce high titers of Ad-IU2 (1.02×10^{12} vp/ml). Ad-IU2 effectively induced apoptosis in receptive, PSA/PSMA-positive cells (Figure 2), and this was correlated to strong *in vitro* killing ability. The addition of TRAIL to a PSRCA augmented the killing power of a replication-competent virus (Figure 3), and Ad-IU2 significantly suppressed the *in vivo* growth of androgen-independent CWR22rv xenografts in nude mice compared to a PSRCA control (3.1 *vs*. 17.1-fold growth of tumor). Important to the clinical success of a PSA/PSMA-restricted replication-competent adenovirus is the ability to target PSA/PSMA-negative prostate cancer cells as well as cells beyond the direct contact of infected cells. This effect was mediated by direct cell-to-cell contact with or soluble factors released from Ad-IU2-infected cells, and its *in vivo* efficacy was evidenced by apoptosis detection away from necrotic centers of viral replication in CWR22rv tumor xenografts (Figure 5H). It is assumed that because the treatment is administered intralesionally, the bystander effect will remain within the local tumor; however, prior to the clinical translation of this molecular therapy, toxicology studies must be performed to determine whether normal cells outside of the prostate are affected. Likewise, it should be determined whether the soluble factors responsible for the local bystander effect have an impact on distant tumor sites.

In summary, we have developed a novel strategy to deliver TRAIL to androgen-independent tumors of the prostate via a prostate-restricted oncolytic adenovirus. The use of the highly tissue-specific PSES promoter will allow systemic administration of the virus to target distant metastases as well as locally advanced or recurrent tumors without damaging vital organs such as the liver. Of the prostate-specific promoters available, PSES retains the highest tissue-specificity and activity in conditions depleted of androgens, giving this PSRCA greater clinical utility in patients undergoing simultaneous ADT. Although gene therapy may not be feasible as a monotherapy for advanced androgen-independent prostate cancer, Ad-IU2 may benefit from combination with conventional therapies such as chemotherapy or radiation therapy.

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Abreviations List

ADT androgen deprivation therapy

TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
FADD	Fas-associated death domain
DISC	death-inducing signaling complex
PSRCA	prostate-specific replication-competent adenovirus
PSES	prostate-specific enhancing sequence
PSA	prostate-specific antigen
PSMA	prostate-specific membrane antigen
PSME	PSMA-enhancer
RSV	Rous sarcoma virus
CMV	cytomegalovirus
HER	human embryonic retinoblast
HDFa	adult human dermal fibroblast
EGFP	enhanced green fluorescent protein
LDU	lethal $dose_{50}$ units
vp	virus particles
FACS	fluorescence-activated cell sorting
PI	prodidium iodide
mRFP	monomeric red fluorescent protein
SQ	subcutaneous
H&E	Hematoxylin and Eosin
TUNEL	terminal dUTP nick-end labeling
hTERT	human telomerase reverse transcriptase

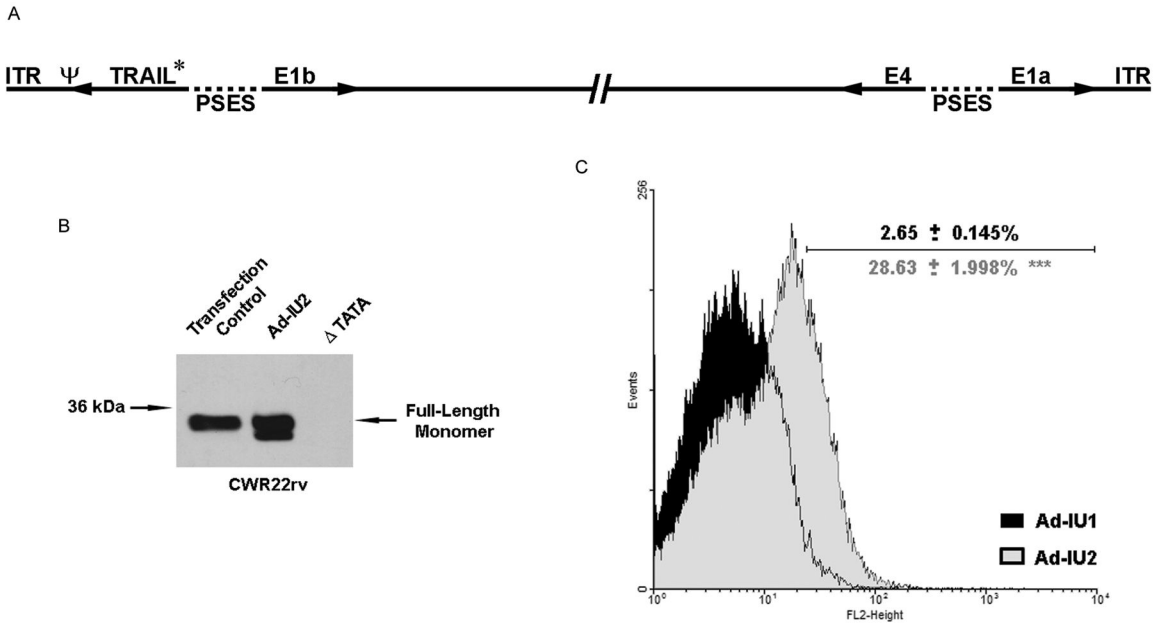

Figure 1.

Characterization of Ad-IU2. **A**, genomic structure of Ad-IU2. TRAIL cDNA was cloned into the left ITR under control of the bidirectional PSES enhancer. To avoid interference with the adenoviral packaging sequence (ψ), *E1a* was placed at the right ITR under the transcriptional control of PSES along with *E4*. Replication competent adenoviral control vector, Ad-IU1 was constructed by replacing the PSES-TRAIL cassette with a PSES-HSV-TK expression cassette (*). **B**, immunoblot confirming TRAIL expression in PSA/PSMA-positive CWR22rv cells following infection with 0.01 LDU/cell Ad-IU2 or transfection with pORF-hTRAIL. No endogenous TRAIL expression was detected following infection with Ad-ΔTATA-E1a. **C**, cell surface expression of TRAIL was confirmed in CWR22rv prostate cancer cells following infection with Ad-IU2. Overlapping histograms for Ad-IU1 and Ad-IU2 are depicted. *** = $p<0.001$.

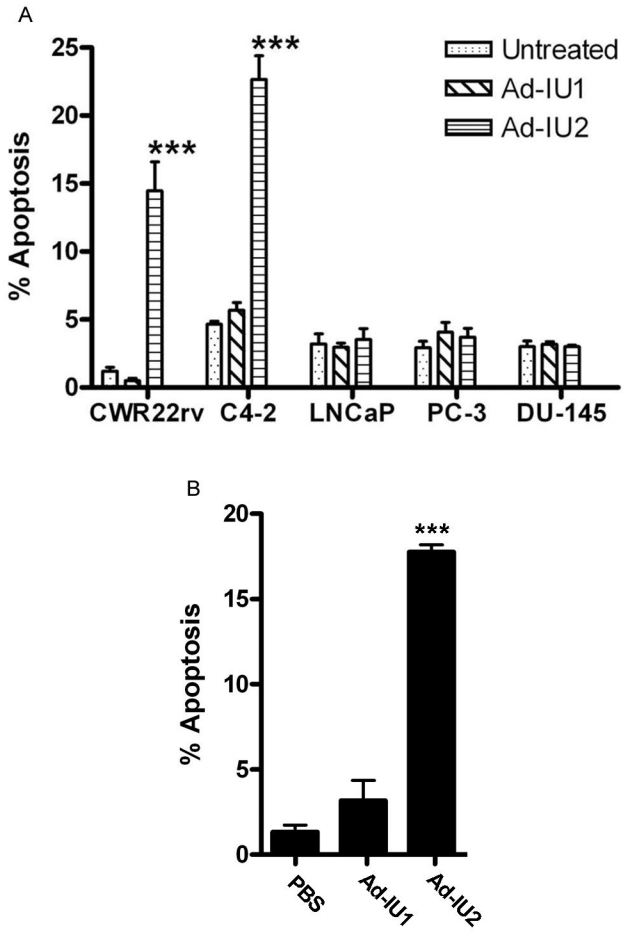

Figure 2.

Apoptosis induction by Ad-IU2. **A**, 0.01 LDU/cell Ad-IU2 induced 5-fold greater apoptosis at 24 hours than the PSRCA control, Ad-IU1, specifically in PSA/PSMA-positive prostate cancer cells. **B**, infection of TRAIL-resistant, PSA/PSMA-positive LNCaP cells with Ad-IU2 for 48 hours reversed resistance to TRAIL-mediated apoptosis, inducing apoptosis to similar levels as in TRAIL-sensitive PSA/PSMA-positive prostate cancer cells. *** = $p<0.001$.

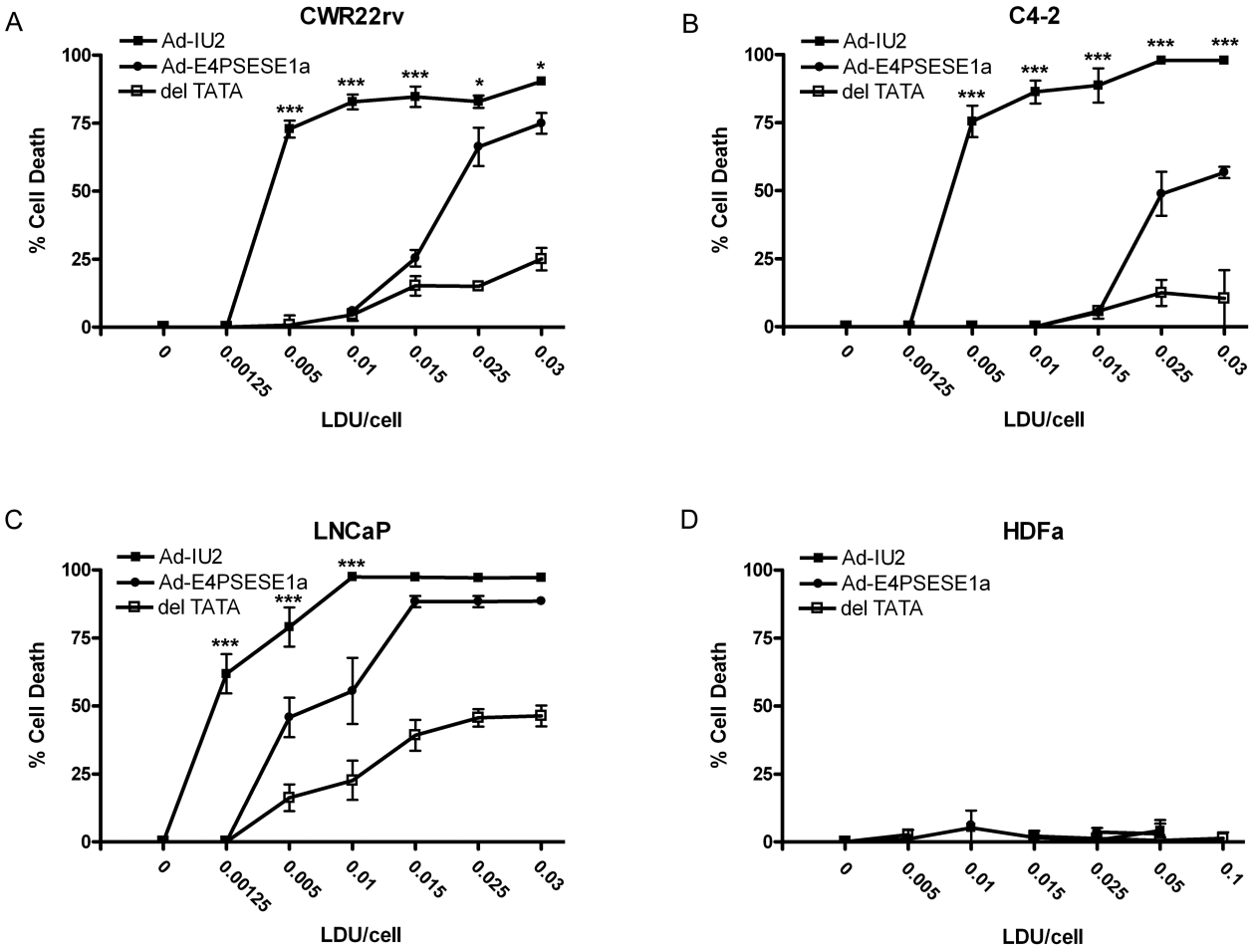

Figure 3.

Cytotoxicity of Ad-IU2 was specific to PSA/PSMA-positive prostate cancer cells. Crystal violet killing curves for CWR22rv (**A**), C4-2 (**B**), LNCaP (**C**) and human dermal fibroblasts (**D**). Cell killing was assayed once cytopathic effect was detected at 4 days (CWR22rv), 3 days (C4-2 and LNCaP) and 7 days post-infection (HDFa, cytopathic effect was not detected). * = $p<0.05$, *** = $p<0.001$ difference between Ad-IU2 and Ad-E4PSESE1a.

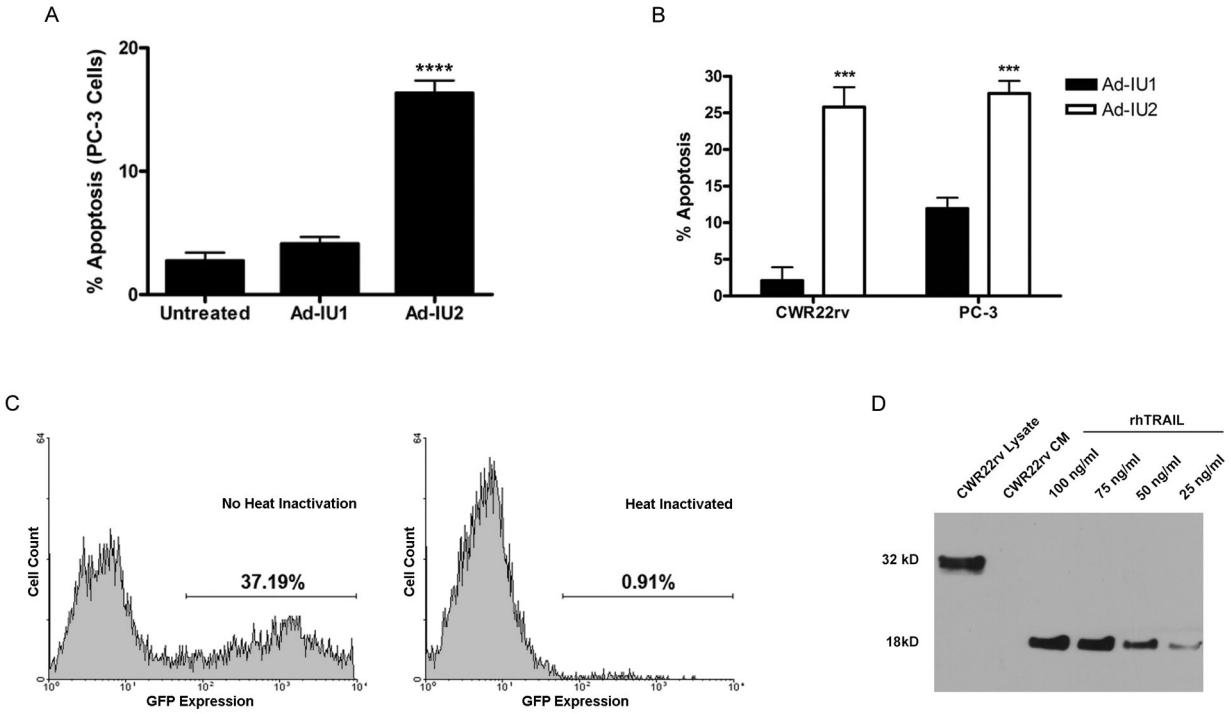

Figure 4.

Bystander effect of Ad-IU2. **A**, marked apoptosis was induced in mRFP-labeled PC-3 cells co-cultured with CWR22rv cells 24 hours after infection with Ad-IU2. **B**, heat-inactivated, apoptotic body-enriched conditioned media from Ad-IU2 infected CWR22rv cells induced significant levels of apoptosis in PSA/PSMA-positive and -negative prostate cancer cells. **C**, heat treatment of conditioned media was sufficient to inactivate adenovirus, as indicated by a drastic reduction in GFP-positive CWR22rv cells following treatment with heat-inactivated Ad-E4PSESE1a conditioned media. **D**, TRAIL was not cleaved from the surface of Ad-IU2-infected CWR22rv cells and present in conditioned medium at physiologically relevant concentrations. CWR22rv cells were infected with 0.01 LDU/cell Ad-IU2 for 48 hours, and cell lysate and conditioned medium (CM) were collected. Cell lysate, CM and various concentrations of soluble rhTRAIL were separated by 12% SDS-PAGE and immunoblotted with anti-human TRAIL antibody. *** = $p<0.001$, **** = $p<0.0001$.

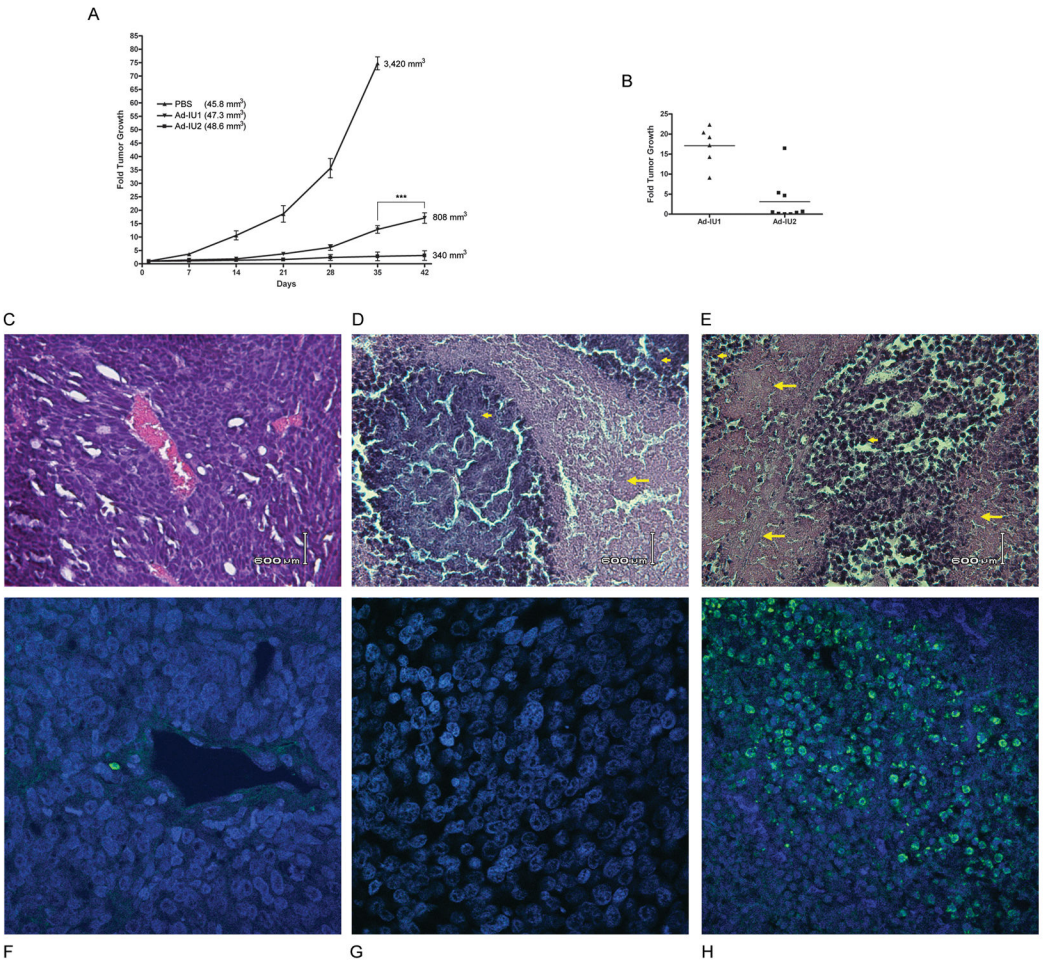

Figure 5.

Ad-IU2 suppressed the growth of androgen-independent human prostate tumors in athymic mice. **A**, subcutaneous androgen-independent CWR22rv xenografts were established in castrated male athymic mice and treated with intratumoral injections of PBS (vehicle control, n = 5), Ad-IU1 (PSRCA control, n = 6) or Ad-IU2 (n = 9). Mean tumor volumes at day 0 and study endpoints are listed. *** = $p<0.001$ (Ad-IU2 *vs*. Ad-IU1). **B**, fold tumor growths for individual mice at the 6-week end-point. Histological appearance of harvested tumors 6 weeks after treatment with PBS (**C**), Ad-IU1 (**D**) (large yellow arrows, necrotic centers of oncolysis; small yellow arrows, patches of healthy tumor cells) or Ad-IU2 (**E**) (large yellow arrows, necrotic centers of oncolysis; small yellow arrows, condensed nuclei) (200× magnification). *In situ* TUNEL assay detected no apoptosis in tumors treated with PBS (**F**) or Ad-IU1 (**G**) and marked apoptosis in tumors treated with Ad-IU2 (**H**).

Table 1

Ad-IU2 replicated efficiently in and restricted to PSA/PSMA-positive prostate cancer cells. Replication assay was performed in HER911E4 producer cells with viral supernatants collected from infected PSA/PSMA-positive (CWR22rv, C4-2 and LNCaP) and PSA/PSMA-negative (PC-3 and DU-145) cells. LD_{50} was calculated as the greatest viral dilution factor producing a cytopathic effect in at least 4 of 8 wells. *Replication-deficient negative control. **Replication-competent negative control.

	Input Dose (LDU)	Output Viral Dose (LD_{50})		
Cell Line		**ΔTATA***	**Ad-E4PSESE1a****	**Ad-IU2**
CWR22rv	1×10^4	3.4×10^2	3.7×10^6	3.7×10^6
C4-2	1×10^4	7×10^1	4×10^5	4×10^5
LNCaP	1×10^4	7×10^1	7×10^5	3.7×10^6
PC-3	1×10^5	7×10^1	4×10^1	4×10^1
DU-145	1×10^5	7×10^1	7×10^1	7×10^1