Fas Ligand Delivery by a Prostate-Restricted Replicative Adenovirus Enhances Safety and Antitumor Efficacy

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Abstract

Purpose: Recent studies showed that Fas ligand (FasL) induced apoptosis in tumor cells and suppressed the immune response in several types of tumors. However, the toxicity of FasL limited further administration. This study delivered FasL in prostate cancer cells using an improved prostate-restricted replicative adenovirus (PRRA), thereby improving the antitumor effect while decreasing systemic toxicity.

Experimental Design: We designed a FasL-armed PRRA, called AdIU3, by placing adenoviral E1a and E4 genes, FasL cDNA, and E1b gene under the control of two individual PSES enhancers. Tissue-specific viral replication and FasL expression were analyzed, and the tumor killing effect of AdIU3 was investigated both in vitro and in vivo using androgen-independent CWR22rv s.c. models via local administration and bone models via systemic administration. The safety of systemic administration of AdIU3 was evaluated. AdCMVFasL, in which FasL was controlled by a universal cytomegalovirus (CMV) promoter, was used as a control.

Results: AdIU3 enhanced FasL expression in prostate-specific antigen (PSA)/prostate-specific membrane antigen (PSMA)-positive cells but not in PSA/PMSA-negative cells. It induced apoptosis and killed PSA/PMSA-positive prostate cancer cells but spared normal human fibroblasts, hepatocytes, and negative cells. The increase in killing activity was confirmed to result in part from a bystander killing effect. Furthermore, AdIU3 was more effective than a plain PRRA in inhibiting the growth of androgen-independent prostate cancer xenografts and bone tumor formation. Importantly, systemic administration of AdIU3 resulted in undetectable toxicity, whereas the same doses of AdCMVFasL killed all mice due to multiviscera failure in 16 h.

Conclusions: AdIU3 decreased the toxicity of FasL by controlling its expression with PSES, with greatly enhanced prostate cancer antitumor efficacy. The results suggested that toxic antitumor factors can be delivered safely by a PRRA.

Prostate cancer is the most frequently diagnosed malignancy and one of the leading causes of cancer death in men. In 2007, an estimated 218,890 American men will be diagnosed with prostate cancer, and 27,050 men will die from this disease. Despite recent advances in the early detection and treatment of locally advanced prostate cancer, the prognosis for patients with advanced prostate cancer is poor. Most patients with advanced prostate cancer respond initially to androgen ablation therapy, but emergence of hormone-refractory disease is inevitable, usually in widespread metastasis with a fatal outcome (1, 2).

One of the best-characterized pathways leading to apoptosis is initiated by the binding of Fas ligand (Fasl) to its receptor Fas. Fas is a 45k Daltons type I membrane protein belonging to the tumor necrosis factor/nerve growth factor superfamily of receptors (3–5), and Fasl is a 40k Daltons type II membrane protein belonging to the tumor necrosis factor family (6, 7). Following engagement with Fasl, Fas initiates an apoptotic signal inside the cell. This signal originates at the death-inducing signaling complex, which forms just below the cell surface on the cytoplasmic domain of Fas. The death-inducing signaling complex, in part, is composed of Fas, an adapter molecule (FADD/MORT), and procaspase-8 (FLICE/MACH; ref. 8). On Fas stimulation, procaspase-8 is automatically activated (9). Activated caspase-8, in turn, cleaves and/or activates several downstream substrates, including the effector caspase-3 and caspase-7 (10). These effector caspases are responsible for the cleavage of vital cellular substrates [e.g., RB, poly(ADP-ribose) polymerase, and lamins], which ultimately leads to apoptosis.

Although the expression of Fas is elevated in prostate cancer (11), it has been shown that prostate cancer and other tumor
types are resistant to Fas/Fasl-mediated apoptosis (11–13). Several laboratories have attempted with little success to induce Fas-mediated apoptosis in prostate cancer cells using different external Fas agonists, such as anti-Fas antibodies and membrane-bound Fasl. (14–16). Overcoming the blockade of the Fas/Fasl. apoptosis pathway in prostate cancer cells is a critical challenge. Gene therapy is a promising approach for the treatment of androgen-independent prostate cancer, if the therapeutic genes can be delivered to enough target cells and expressed at a sufficient level and duration. Most prostate cancer cell lines respond best to Fasl. when it is expressed following delivery by adenovirus (17, 18). However, initial studies using replication-deficient adenovirus to express Fasl. for the treatment of prostate cancer yielded limited success.

Two limitations hindered the success of early Fasl.-based cancer gene therapies. First, a replication-deficient adenovirus has poor in vivo gene delivery efficiency. Second, the use of a universal cytomegalovirus (CMV) promoter limits its application to intratumoral injection because Fasl. induces hepatocyte apoptosis and liver failure (19, 20). We explored the combination of Fasl. and a prostate-restricted replicative adenovirus (PRRA) to enhance in vivo gene delivery efficiency while using a strong prostate-specific enhancer, PSES, to drive the expression of Fasl. and decrease undesired liver toxicity, which allows systemic administration.

Materials and Methods

Cells and cell culture. HER911E4 (a gift from Dr. Bert Vogelstein, John Hopkins, Baltimore, MD) is a HER911 derivative that expresses adenoviral E4 protein under the control of tetR promoter (21). HER911E4 was maintained in DMEM supplemented with 0.1 mg/mL hygromycin B (Calbiochem) and 2 μg/mL doxycycline (Sigma). Prostate cancer cells C4-2, CWR22rv, PC-3, and DU-145 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. CWR22rv-RFP was established by the stable transfection of CWR22rv cells with red fluorescent protein gene followed by G418 cell cloning selection (data not shown). Human dermal fibroblast was cultured in medium 106 (Cascade Biologics). Human fetal hepatocyte hNHeps (Cambrex) was cultured in Eagle’s MEM (American Type Culture Collection). All cells were fed two to three times per week with fresh growth medium and maintained at 37°C in a 5% CO2 incubator.

Construction of recombinant adenovirus. The recombinant adenovirus construction strategy is based on a system developed by Dr. Xavier Danthine (O.D.260, Inc., Boise, ID) and was similar to that for AdE4PSESE1a (22). Additionally, a PSES-Fasl expression cassette was cloned into pAd1020 to make pAd1020PSES-Fasl, which was then digested with SfiI to release PSES-Fasl together with the adenoviral left inverted terminal repeat and packaging signal (1-358 bp) and a kanamycin resistance gene (Kan). The Kan- inverted terminal repeat-FasL-PSES fragment was then cloned into SfiI-digested pAd288E1E4PSESE1a. The ligated DNA was transformed into Escherichia coli cells, which were then plated onto agar plates containing both ampicillin and kanamycin. Cosmid DNA was purified, digested with Pac1 to release adenoviral genome, and transfected into HER911E4 using a LipofectAMINE 2000 transfection reagent (Invitrogen) to generate AdlIU3. The total length of the recombinant viral genome is 37,953 bp. This structure is illustrated in Fig. 1. The package and titration methods of AdlIU3 are the same as AdE4PSESE1a (22). The viral DNA was purified by phenol/chloroform extraction and used as a template for PCR. Four PCR primers were chosen within the Fasl.PSES-E1b gene cassette, including Fasl. reverse (primer 1: TGTCTCCTCCCTCCTCCTCAG), PSES forward (primer 2: GAGAGAAAACATATTTATTTACGA), PSES reverse (primer 3: AGATCTCCGATGACGTTAAATGTGAT), and E1b reverse (primer 4: AGATGGTTTCTGCTGTCATT). Two other PCR primers were chosen within the E4-PSES-E1a gene cassette, including E4 reverse (primer 5: ACCACTCGGCTAGCCGAAAAATTGCCACCT) and E1a reverse (primer 6: CGGGAAAAATCTCGCAGAAC). Two other control adenoviruses were constructed. AdlIU1 has a similar structure to AdlIU3, but the Fasl. CDNA is replaced with a thymidine kinase gene from herpes simplex virus. The second control virus is AdCMVFasl., an E1/E3-deleted replication-deficient adenovirus with CMV-Fasl. expression cassette. The apoptosis of Fasl/Fasl was blocked by a pan-spectrum caspase inhibitor, Z-VAD-FMK, in the production and amplification of AdCMVFasl.

Western blot analysis. C4-2, fibroblast, and hNHeps cells were seeded in P100 dishes and infected with AdlIU3 and AdCMVFasl. 1 day after cell seeding. C4-2 was infected with 66 vsv particles (v.p.) per cell, fibroblast with 1,000 v.p. per cell, and hNHeps with 462 v.p. per cell for a similar infectivity (22). The supernatant was used for the preparation of conditioned medium (23). The same doses of protein (40 μg) in conditioned medium were subjected to SDS-PAGE separation and electroblotted to a nitrocellular membrane. The membrane was probed with an anti-Fasl. antibody (Santa Cruz Biotechnology, Inc.) followed by a horseradish peroxidase–conjugated anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology). SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used to detect the signal.

Flow cytometric analysis (fluorescence-activated cell sorting) to detect membrane Fasl. expression. C4-2, fibroblast, and hNHeps cells were seeded in 12-well plates (5 × 10^5 per well) and infected with AdIU1 or AdCMVFasl. 1 day after cell seeding, AdlIU1 and PBS were used as controls. Each cell line was infected with standardized virus doses (22). Cells were harvested using 0.25% trypsin 24 h after infection, washed with fluorescence-activated cell sorting (FACS) buffer (PBS with 5% fetal bovine serum and 0.1% sodium azide) on ice, resuspended in 50 to 100 μL of buffer, and incubated on ice for 30 min with anti-Fasl. antibody (1:20 dilution; Apex, Inc.) followed by three washings. Then, the cells were incubated with a fluorescein-conjugated IgG secondary antibody (1:100 dilution; Apex) for 30 min followed by three washings. Finally, the cells were fixed in 0.5 to 1 mL of cold 1% paraformaldehyde solution for FACS analysis.

FACS analysis to detect apoptosis induction ability. CWR22rv, LNCaP, C4-2, DU-145, fibroblast, and hNHeps cells were seeded in 12-well plates (5 × 10^5 per well) and infected with AdlIU1 1 day after cell seeding with AdIU1 or PBS as negative controls. Each cell line was infected with standardized virus doses as described above. Cells were harvested with 0.25% trypsin 48 h after virus infection and washed with PBS once. An Annexin V/proidium iodide apoptosis detection kit (BD Biotechnology) was used to detect apoptosis by FACS analysis.

Viral replication assay. C4-2, CWR22rv, PC-3, DU-145, fibroblast, and hNHeps cells were seeded in six-well plates (1 × 10^5 per well) 1 day before viral infection and subsequently infected with AdlIU3 or AdlIU1. Each cell line was infected with standardized doses of virus for similar infectivity [input doses: C4-2, 6.6 × 10^4 infectious forming units (IFU); CWR22rv, 2 × 10^5 IFU; PC-3, 2.3 × 10^5 IFU; DU-145, 1.6 × 10^5 IFU; fibroblast, 1.6 × 10^5 IFU; and hNHeps, 4.6 × 10^5 IFU]. The media were changed 8 h later. The cells were harvested and subjected to three freeze/thaw cycles 2 days after virus infection. Virus supernatants were harvested and titrated by virus titrature assay. Briefly, HER911E4 cells were seeded in 96-well plates (5 × 10^4 per well) 1 day before viral infection. The cells were infected with serial volume dilutions of the harvested supernatant ranging from 1 to 10^{-11} μL per well. A row of eight wells was used for each dose. The media were changed on day 4, and the cells were examined under the microscope on day 7. The doses of the produced viruses were shown as a multiplication of the total soup volume and a L_50 value (the dilution factor that causes a cytotoxic effect in at least four wells of cells in a row on a 96-well plate on day 7). The titer assay was done twice and averaged.
**Crystal violet cell killing assay.** CWR22rv, fibroblast, and hNHeps cells were seeded in 24-well plates (1 × 10^5 per well) 1 day before infection. CWR22rv cells were infected with serial doses ranging from 0.2 to 2,000 v.p. per cell of AdE4PSESE1a, AdIU3, or AdIU1. Fibroblasts and hNHeps were infected with wild-type adenovirus, AdIU3, or AdIU1 at the same diluted doses. A row of six wells was used for each virus. The growth media were changed on every other day. Each cell line was evaluated individually. Cells were monitored under the light microscope daily. Crystal violet staining was then done to detect living cells at day 7 after virus infection (24).

**Bystander killing assay.** CWR22rv cells were seeded in a 24-well plate (2.5 × 10^5 per well) 1 day before virus infection. The cells were infected with 100 v.p. per cell of AdIU3 or AdIU1 for 6 h, with uninfected cells as a control. The cells were washed thrice by PBS to clear away all residual viruses and cocultured with CWR22rv-RFP at a mixture ratio of 10:1 for an additional 16 h. The cells were observed under an inverted fluorescent microscope following harvesting and stained with FITC-Annexin V for FACS analysis. Triplicate samples were done for the statistics evaluation.

**Apoptosis induction of virus-inactivated conditioned medium.** The remaining adenoviruses in the conditioned medium were inactivated by heating the conditioned medium collected from AdIU3-infected C4-2 cells at 56°C for 30 min, which can be confirmed by the disappearance of virus-injected green fluorescent cells for AdE4PSESE1a (the virus was used as a control in the process). Conditioned media (300 μg) were used to treat prostate-specific antigen (PSA)/prostate-specific membrane antigen (PSMA)–positive C4-2 and PSA/PSMA-negative fibroblast cells for 48 h, and the apoptotic cells were counted by FACS analysis as described above.

**Evaluation of therapeutic efficacy in s.c. tumor xenografts.** All animal methods and procedures were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. CWR22rv tumors were established by injecting 2 × 10^6 cells s.c. in the flanks of athymic nude mice (6-week-old males). The injected mice were castrated 3 days after cellular injection. Mice with similar tumor sizes (~50 mm^3) were randomized 3 weeks after cell inoculation (eight tumors in six mice each group) and treated with 1 × 10^10 v.p. of AdIU3, AdE4PSESE1a, or AdCMVGFP (a replication-deficient adenovirus used as a negative control) in 100 μL PBS via intratumoral injections. Tumor sizes were measured once every week with calipers, and the following formula was applied to calculate tumor volume: length × width^2 × 0.5236 (25). Tumor growth curves were drawn according to the weekly measurements. Significant differences between treatment and control groups were analyzed using Student’s t test.

**Evaluation of therapeutic efficacy in bone tumors.** To evaluate the effect of systemic AdIU3 in the intraosseous prostate tumor model, 1 × 10^6 of CWR22rv cells were injected into the bone marrow space of double tibias in castrated male nude mice according to procedures published previously (26). One week after induction, a dose of 5 × 10^10 v.p. AdIU3 in 100 μL PBS was given i.v. to the mice twice at 1-week intervals, with AdCMVGFP and AdE4PSESE1a as controls. AdCMVGFP- and AdE4PSESE1a-treated mice showed leg swelling, and the swelling aggravated sharply at 12 weeks after virus injections. At this time, X-ray images were taken before sacrifice.

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**Fig. 1.** AdIU3 delivered highly specific FasL expression in PSA/PSMA-positive prostate cancer cells. A, schematic illustration of AdIU3. Adenoviral E3a and E4 genes and FasL cDNA are under the control of two copies of PSES enhancers. Gene structure of AdIU3 is confirmed by PCR. ITR, inverted terminal repeat. B, AdIU3- or AdCMVFasL-infected C4-2 and fibroblast cells were harvested for Western blot analysis. C, membrane FasL expressions on AdIU3- or AdCMVFasL-infected C4-2, fibroblast, and hNHeps were analyzed by FACS with an anti-FasL polyclonal antibody and a fluorescein-conjugated IgG secondary antibody. AdIU3 specifically induced FasL expression in PSA/PSMA-positive C4-2 cells but only faint expression in PSA/PSMA-negative fibroblast and hepatocyte cells. AdCMVFasL induced FasL expression in all cell lines.
Safety evaluation of AdIU3 via systemic administration. Animals were subjected to tail vein injection of 5 × 10⁶ v.p. (a dose used for bone models above) of AdIU3 or AdCMVFasL in 100 μL PBS (six mice per group). All mice treated by AdCMVFasL died within 16 h of virus injection. Heart, liver, spleen, lung, kidney, stomach, and carotid artery were collected and fixed in formalin for histologic examination. Other animals were kept until 32 days, and the liver functions were evaluated (including relative liver weight, aspartate aminotransferase, and alanine aminotransferase).

Histology, immunohistochemistry, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. Tumors and visera were removed, immediately fixed in formalin, and embedded in paraffin. Tissue sections were stained with H&E. For immunologic detection of adenovirus 5, a polyclonal rabbit antibody to adenovirus type 5 (Abcam) and a biotinylated polyclonal anti-rabbit antibody were applied. For detection of FasL, a polyclonal rabbit antibody to FasL (Santa Cruz Biotechnology) and a biotinylated polyclonal anti-rabbit antibody (Santa Cruz Biotechnology) were applied. The in situ apoptosis detection kit was purchased from Roche Diagnostics. The protocol followed the manufacturer's manual. The dark brown nuclear staining cells were counted in 10 randomly selected vision fields (×20), and the difference was compared among three groups. Results were expressed as mean ± SD of 10 measurements. Statistical analyses were two sided and done at the α = 0.05 statistical significance level.

Results

AdIU3 is a novel FasL-armed PRRA. We have designed a novel strategy to control adenovirus replication and therapeutic gene expression using two copies of tissue/tumor-specific promoters/enhancers in a single adenoviral backbone. The bidirectional prostate-specific enhancer, PSES, was placed between the E1a and E4 genes to direct their expression so as to control adenovirus replication tightly. In addition, a second copy of PSES was placed at the left end of the adenovirus to direct FasL expression. The gene structure of AdIU3 was confirmed by PCR using genomic DNA extracted from AdIU3-infected 911E4 cells. Primers were chosen in the left FasL-PSES-E1b region and the right E4-PSES-E1a region. All PCR products have the expected sizes, indicating that no gross rearrangement occurred during virus production. A negative control in FACS and cell coculture experiments (AdE4PSESE1a-infected cells show green color), we constructed another plain PRRA, AdIU1, in which herpes simplex virus-thymidine kinase gene replaces FasL. Alternately, for the toxicity evaluation of AdIU3, we made another control replication-deficient virus, AdCMVFasL, in which FasL expression is driven by a CMV promoter (Fig. 1A).

To determine whether FasL expression can be controlled in a prostate-specific fashion, we analyzed FasL expression in AdIU3- or AdCMVFasL-infected cells by both Western blot and flow cytometry analysis. The result of Western blot shows that, in AdIU3, FasL is under tight control of PSES. Thus, protein expression was detected only in PSA/PSMA-positive C4-2, and the expression increased with time. Only very faint expressions were detected in PSA/PSMA-negative normal human fibroblast and human hepatocyte. In AdCMVFasL, FasL is under the control of a universal CMV promoter and similar FasL expression was detected in both PSA/PSMA-positive and PSA/PSMA-negative cells. The expression did not increase significantly with time because the virus is replication deficient (Fig. 1B). Alternately, membrane FasL expression on the cell surfaces was analyzed by FACS. As expected, FasL expression on the three cell lines remained at the same basal level as PBS when cells were infected with AdIU1, which suggested that adenoviral infection itself has no effect on FasL expression. However, FasL expression was enhanced significantly at 32.06% and specifically in PSA/PSMA-positive C4-2 when the cells were infected by AdIU3. There was only minimum enhancement in PSA/PSMA-negative hepatocyte and fibroblast. Consistent with the results of Western blot, 36.28%, 26.7%, and 32.13% enhancements were detected on AdCMVFasL-infected C4-2, fibroblast, and hepatocyte cells (Fig. 1C).

Because FasL is a death ligand, a high level of FasL expression in the virus-infected cell might stimulate premature cell apoptosis, which could interfere with viral replication and diminish viral production efficiency (27). We did an in vitro viral replication assay to compare viral replication efficiency. As shown in Table 1, AdIU3 propagated as efficiently as AdIU1 in PSA/PSMA-positive C4-2, and CWR22rv cells, and its replication activity was limited in PSA/PSMA-negative prostate cancer cells, human normal fibroblasts, and hepatocytes. Our study has proved that the insertion of a FasL expression cassette did not adversely affect the prostate cancer-specific replication of PRRA.

AdIU3 showed strong tissue/tumor-specific apoptosis and killing abilities. Figure 2A shows that AdIU3 induced a significantly higher percentage of apoptotic cells than AdIU1 in the PSA/PSMA-positive C4-2, LNCaP, and CWR22rv cells (P < 0.05) but did not affect PSA/PSMA-negative DU-145, fibroblast, and hNHeps cells. The result indicated that the incorporation of FasL enhanced apoptosis to a greater degree than a plain PRRA in PSA/PSMA-positive prostate cancer cells, and no significantly higher degree of apoptosis was found between AdIU1- and AdIU3-infected hepatocytes (P > 0.05). Although hepatocytes express Fas receptor abundantly and are especially susceptible to damage by FasL, the apoptosis induction of AdIU3 is limited because the levels of FasL delivered by PRRA are too low to activate Fas/FasL-mediated apoptosis programs in hepatocytes. Consistently, the killing activity of AdIU3 increased 10-fold over AdE4PSESE1a and 100-fold over AdIU1 in CWR22rv cells. Conversely, cell killing activities were dramatically attenuated in PSA/PSMA-negative DU-145, fibroblast, and hepatocyte cells (Fig. 2B).
in fibroblasts and human hepatocytes, far lower than that of wild-type adenovirus [Fig. 2B, killing activity of AdE4PSESE1a toward CWR22rv cell was similar to wild-type adenovirus (22)].

The higher killing activity is due to a bystander effect probably from the apoptotic vesicles. The virus was cleared away, and AdIU3-infected CWR22rv cells were cocultured with the red CWR22rv-RFP Cell. The majority of red cells were normal when they were cocultured with PBS-treated CWR22rv cells, and some red cells became round when cocultured with AdIU1-infected CWR22rv cells, whereas the majority of red cells rounded when cocultured with AdIU3-infected CWR22rv cells. Compared with the apoptotic red cell percentage in the PBS-treated cells, FACS analysis detected a 24.36% increase in percentages of apoptotic red cells in the AdIU3-treated cells but only a 2.28% increased percentage of apoptotic cells in the AdIU1-treated cells ($P < 0.001$; Fig. 3A). These data suggested that the enhanced cell killing resulted from a Fas/FasL-mediated bystander effect.

To clarify whether the bystander effect of AdIU3 is from the cleaved soluble FasL or from possible apoptotic vesicles following the virus infection, we tested the conditioned medium from virus-infected CWR22rv cells. In contrast to the conditioned medium from PBS-treated or AdIU1-infected cells, the conditioned medium from AdIU3-infected cells induced many more apoptotic cells in both PSA/PSMA-positive C4-2 and PSA/PSMA-negative fibroblast and hNHeps cells. We did not detect membrane or soluble FasL protein expressions in the conditioned medium by Western blot (Fig. 3B), which indicated that the bystander effect of AdIU3 is not confined to membrane FasL protein on the surface of AdIU3-infected CWR22rv but also possibly is from the apoptotic vesicles generated by AdIU3-infected tumor cells in the conditioned medium.
AdIU3 retarded the growth of androgen-independent prostate cancer s.c. models more effectively than a plain PRRA. CWR22rv s.c. tumors were established in athymic nude mice. Figure 4A shows that AdIU3 and AdE4PSESE1a significantly diminished tumor growth compared with AdCMVGFP, a replication-deficient adenovirus, and AdIU3 inhibited tumor growth more effectively than AdE4PSESE1a due to the addition of FasL. Two tumors from two animals regressed completely in the AdIU3-treated group.

When s.c. tumors were harvested for histologic examination, tumor masses were small with some fragmentary and necrotic cotton-shaped tissues embedded in a turbid liquid. Tumor histology revealed that small, patchy island-shaped tumor tissues were surrounded by extensive necrotic tissue inside the tumors in the AdIU3-treated group, similar to AdE4PSESE1a-treated tumors (H&E staining data not shown). In the AdCMVGFP-treated group, the tumors were large and evenly solid (22). Anti-adenovirus 5 immunohistochemical staining exhibited extensive viral infection throughout both AdE4PSESE1a- and AdIU3-treated tumors, mainly in tumor cells at the border between tumor and necrosis, similar to the extent in AdE4PSESE1a-treated tumors (P > 0.05). A large number of anti-FasL positive staining cells can be detected inside AdIU3-infected tumors (Fig. 4B).

In situ terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays were done to detect apoptotic cells in the AdIU3- and AdE4PSESE1a-treated tumors. Dark brown nuclear staining cells were found extensively inside and around the tumor mass in the AdIU3-treated tumors. In contrast, positive staining cells were found only bordering the tumor and necrotic areas where viral replication occurred in AdE4PSESE1a-treated tumors. The number of positive staining cells in AdIU3-treated tumors was significantly higher than that in AdE4PSESE1a-treated tumors (P < 0.05, paired Student's t test; Fig. 4B). These results suggested that programmed cell death was involved in the process of tumor killing by both AdIU3 and AdE4PSESE1a and that AdIU3 had a much stronger killing effect than AdE4PSESE1a, probably due to a bystander killing effect on distant tumor cells from Fas/FasL-mediated apoptotic vesicles.

AdIU3 inhibited the growth of androgen-independent prostate cancer bone models more effectively than a plain PRRA. We evaluated the antitumor efficacy of AdIU3 in CWR22rv osseous tumor models via systemic administration. AdCMVGFP- and AdE4PSESE1a-treated mice showed swelling legs, and the swelling aggravated sharply at 12 weeks after virus injections. X-ray images observed severe bone damage in AdCMVGFP-treated mice, middle-degree bone damage in AdE4PSESE1a-treated mice, and no detected bone damage in AdIU3-treated mice. The histology observed huge-sized tumor masses attaching the damaged bone in AdCMVGFP-treated mice, smaller-sized tumor masses in the damaged bone in AdE4PSESE1a-treated mice.
mice, and no tumor or damaged bone were found in AdIU3-treated mice (Fig. 5). We detected Ad5 antibody-labeled virus-infected tumor cells and TUNEL-positive cells only in the bone sections treated by AdE4PSESE1a (TUNEL data not shown), which provided direct evidence to show virus infection and distribution via systemic administration, but we did not detect positive cells in either AdCMVGFP- or AdIU3-treated leg sections. The absence of virus-infected and apoptotic cells in

![Graphs showing tumor volume over weeks for different treatment groups.](image)

Fig. 4. Antitumor efficacy of local administration of AdIU3 in s.c. androgen-independent prostate cancer tumors. CWR22rv s.c. models were established in athymic nude mice. Mice were randomized 3 wks after cell inoculation and treated by AdIU3, AdE4PSESE1a, or AdCMVGFP via intratumoral injections. Tumor sizes were monitored once weekly. A, average tumor sizes and individual tumor sizes in three treatment groups. The result shows a superior antitumor effect of AdIU3 to a plain PRRA, AdE4PSESE1a. B, histologic representations of virus-treated s.c. tumors. Immunohistochemical staining detected a similar amount of adenovirus 5–positive tumor cells bordering between tumor and necrosis in both AdIU3- and AdE4PSESE1a-treated tumors (P > 0.05). FasL-positive cells were found only in AdIU3-treated tumors. In situ TUNEL assays revealed that apoptotic cells existed extensively both inside and around tumor mass in the AdIU3-treated tumors. In contrast, apoptotic cells existed only in cells bordering the tumor and necrosis areas in the AdE4PSESE1a-treated tumors (P < 0.05). Magnification, ×40. These results suggested that AdIU3 induces a more profound apoptosis effect deep into the tumor mass, likely via a bystander killing effect of Fas/FasL-mediated apoptosis.
the two groups is due to the low viral infection efficiency of AdCMVGFP and the fact that no tumor was found in the AdIU3-treated mice. Systemic treatment with AdIU3 significantly inhibited tumor development compared with the control viruses.

**AdIU3 enhanced the safety of FasL significantly when it was systemically given.** No animal died from systemic injection of $5 \times 10^{10}$ v.p. of AdIU3 in the treatment of osseous tumors or in the toxicity experiment, and the liver function of mice (including aspartate aminotransferase, and alanine aminotransferase) is similar to that of PBS-treated mice at 32 days after virus injection (Table 2). However, at the same dose of AdCMVFasL, all mice died in just 16 h after i.v. injection (Fig. 6A). The pathologic changes in the livers included hepatocyte dissociation, nuclei shrinkage, vast multifocal cellular necrosis, and bleeding, which suggested acute hepatic damage and necrosis, and centrovein congestion suggesting circulation failure. The acute damage to heart muscle included heart muscle fiber fracture and strait disappearance, focal bleeding and capillary stasis, cell acidophilic change, and nuclei condensation in 80% of cells. The lungs showed focal bleeding, collapse or blood congestion, and stromal edema. The kidneys showed tubule epithelial degeneration and capillary stasis, suggesting a blood circulation disorder (data not shown). No detected pathologic change was found in the organs of AdIU3-treated mice 32 days following AdIU3 treatment. Some positive staining cells by adenovirus type 5 or anti-FasL were scattered in the liver, lung, and heart tissues of AdIU3-treated animals. A large amount of positive staining cells stained by adenovirus type 5 or anti-FasL could be detected in the pathologic areas in the liver, lung, and heart tissues of AdCMVFasL-treated animals. Significantly more programmed death was detected by TUNEL assay in the liver, lung, and heart tissues of AdCMVFasL-injected mice than in the livers of AdIU3-injected mice (Fig. 6B).

**Discussion**

Fas/FasL-mediated apoptosis is an important antitumor response used by the immune system to control tumor development and progression and thus presents an attractive strategy for cancer therapy. FasL delivered by adenoviral vector could extend transgene expression (28) and overcome the resistance to Fas antibody-mediated apoptosis in prostate cancer cell lines (29). However, high levels of FasL expression induce hepatocyte apoptosis and liver failure (30). Thus, restricting the specificity of adenoviral FasL gene expression is essential to ensure the safety of FasL (31–33).

We developed a novel adenovirus, AdIU3, by controlling adenovirus replication and FasL expression with two individual PSES enhancers. PSES is a novel prostate-specific enhancer sequence composing of enhancer elements from PSA and PSMA genes (34). PSES can drive adenovirus replication strictly in PSA/PSMA-positive prostate cancer cells by controlling both...
E1a and E4 gene expression (22). AdIU3 only replicates in PSA/PSMA-positive prostate cancer cells, and the replication ability is similar to a plain adenovirus AdIU1, which suggested that FasL, a death ligand, did not inhibit virus replication. In addition, FasL expression is also restricted to the PSA/PSMA-positive prostate cancer cells, and the expression increases with the virus replication, with only very faint expression in normal fibroblast cells and hepatocytes. Consistent with FasL expression, AdIU3 showed much stronger killing and apoptosis induction activities than AdIU1 in PSA/PSMA-positive prostate cancer cells. The stronger activities of AdIU3 are due to the addition of Fas/FasL-mediated apoptosis. Alternatively, the tissue specificity of AdIU3 limited the apoptosis induction and killing ability of AdIU3 in hepatocytes, although they express receptor Fas abundantly and are especially susceptible to damage by FasL. Therefore, with the double control of PSES, AdIU3 diminished significantly the toxicity in vivo when the therapeutic doses were given i.v. However, at the same doses, the replication-deficient adenovirus AdCMVFasL, in which FasL is controlled by a CMV promoter, killed all animals in just 16 h after virus injection. Failure of multiple viscera due to liver failure was found. High level of adenovirus infection and FasL expression and severe cell apoptosis were detected in such organs as liver, lung, and heart tissues by immunohistochemical staining.

Clearing away the effect of adenovirus, we found that more neighboring virus-uninfected prostate cancer cells experienced

Fig. 6. Safety evaluation of AdIU3 via systemic administration. Animals received $5 \times 10^{10}$ v.p. of AdIU3 or AdCMVFasL treatment via i.v. injection. A, mice treated by AdCMVFasL died in just 6 h and all mice died within 16 h after virus injection, whereas mice treated by AdIU3 survived with no damage detected in liver function in the experimental time window. The result showed that toxicity of FasL for normal tissues diminished significantly when it was delivered by a PRRA. B, organs were harvested for histopathology 32 d after virus injection. The representative pathology in AdCMVFasL-treated livers, lung, and heart indicated multiple viscera failure. Weak expression of adenovirus proteins and FasL could be detected in the livers, lungs, and hearts of animals treated with AdIU3. On the other hand, the much stronger expression of adenovirus proteins and FasL in animals treated by AdCMVFasL was likely due to the influence of the strong CMV promoter. Consistent with FasL expression, TUNEL assay detected strong apoptotic signals in AdCMVFasL-treated livers, lungs, and hearts but not in AdIU3-treated livers, lungs, and hearts. These results suggest that AdIU3 may be safely used to treat cancers via systemic administration.

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Research.
apoptosis when they were cocultured with AdIU3-infected prostate cancer cells. This phenomenon is called a bystander killing effect. Even when the viruses were inactivated in conditioned medium and we did not detect FasL protein expression, the conditioned medium still induced apoptosis in both PSA/PSMA-positive prostate cancer cells and normal fibroblast cells. The data suggested that the bystander killing effect of AdIU3 is derived not only from membrane FasL expressed on the surface of cells but also from the possible apoptotic vesicles and cellular debris produced by AdIU3-infected tumor cells (35–37). FasL microvesicles remain apoptotic vesicles and cellular debris produced by AdIU3-effect of AdIU3 is derived not only from membrane FasL expression, the conditioned medium still induced apoptosis in prostate cancer cells. This phenomenon is called a bystander apoptosis when they were cocultured with AdIU3-infected tumors. However, apoptotic cells could only be found bordering the tumor and necrotic areas in the AdE4PSESE1a-treated tumors, indicating that the control oncolytic adenovirus was unable to kill tumor cells located deep within the tumor nodules due to the limited virus distribution ability. This result strongly suggested that apoptosis induction by AdIU3 leads to a much stronger killing activity than AdE4PSESE1a. The possible mechanism is that FasL microvesicles produced by AdIU3 infection can be distributed more extensively than the viruses and make the apoptosis induction effect continuous inside the tumor mass (38).

In summary, we developed an apoptosis-based PRRA, AdIU3, by controlling adenovirus replication and FasL expression under the control of two copies of PSES. Our novel gene therapy strategy achieved two goals: (a) we enhanced the antitumor efficacy of PRRA by the combination of FasL with an oncolytic adenovirus and (b) we diminished the hepatotoxicity of FasL by putting it under the control of PSES enhancer. This strategy suggests that toxic antitumor factors can be used safely using a PRRA for delivery, and the combination therapeutic modality showed excellent potential for future clinical administration.

### Table 2. Liver function in mice treated by AdIU3

<table>
<thead>
<tr>
<th>Group</th>
<th>Relative liver weight (mean ± SD)</th>
<th>ALT, units/L (mean ± SD)</th>
<th>AST, units/L (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>5.3 ± 0.2</td>
<td>13 ± 9.7</td>
<td>24.7 ± 10.1</td>
</tr>
<tr>
<td>AdE4PSESE1a</td>
<td>5.4 ± 0.3</td>
<td>8.3 ± 1.7</td>
<td>20.7 ± 7.0*</td>
</tr>
<tr>
<td>AdIU3</td>
<td>5.1 ± 0.4</td>
<td>8.5 ± 2.4</td>
<td>19.4 ± 3.8*</td>
</tr>
</tbody>
</table>

*P < 0.05.

### References

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Xiong Li, You-Hong Liu, Yan-Ping Zhang, et al.


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