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ARTICLE

Targeting Prostate Cancer with Conditionally Replicative Adenovirus Using PSMA Enhancer

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Prostate cancer is the second most commonly diagnosed cancer in men and accounts for significant mortality and morbidity in the United States. Initially androgen-dependent, prostate cancer ultimately becomes androgen-independent, which makes the disease extremely difficult to cure. In this study, we examined the use of conditionally replication-competent adenovirus for the treatment of hormone-independent prostate cancer. We utilized PSME, an enhancer element for prostate-specific PSMA expression, to control viral E1A protein expression and achieve exclusive virus replication in prostate. Western blotting confirmed that PSME mediated high E1A protein expression in PSMA-positive, androgen-independent prostate cancer cells (C4-2 and CWR22rv), but was much less active in PSMA-negative cancer cells (PC-3 and A549). Consistent with E1A protein expression, the recombinant adenovirus Ad5-PSME-E1a replicated in C4-2 and CWR22rv almost as efficiently as wild type with low levels of androgen, but its replication was significantly attenuated in PSMA-negative cells. In the *in vitro* killing assay, Ad5-PSME-E1a lysed all C4-2 and CWR22rv cells 5 days after infection, with minimal effect on PSMA-negative cells. In addition, injections of 1.7×10^8 plaque-forming units in a CWR22rv xenograft model in nude mice induced significant tumor growth delay, with a substantial necrotic area. These studies suggest that PSME-driven replication-competent adenovirus may be a new therapeutic modality for prostate cancer patients after hormone ablation therapy.

INTRODUCTION

Prostate cancer remains the second most common solid tumor in men and accounts for significant morbidity and mortality in the United States [1]. This disease, which involves alterations in the expression of growth factors, tumor suppressor genes, and androgen receptor (AR) [2–5], is initially dependent on androgen. Its androgen dependence has been used to slow tumor growth by several means: castration, the use of diethylstilbestrol or luteinizing hormone-releasing hormone agonist to lower circulating serum androgen, and antagonizing androgen action with flutamide, cyproterone acetate, or Casodex. Tumor regression, however, is temporary and the disease evolves to an androgen-independent status [6,7]. Unfortunately, no effective therapy is yet available for androgen-independent prostate cancer patients.

Gene therapy approaches have emerged as an alternative or adjuvant therapeutic modality for prostate cancer patients. The transfer of genetic information to cells *in situ* is accomplished by liposomes or viruses. The prevailing virus-based *in vivo* approaches are: (1) to deliver transgenes such as suicide genes [8,9], tumor suppressor genes [10,11], anti-angiogenesis genes [12], and immune modulators [13] into tumor cells or (2) to lyse tumor cells by tissue- or tumor-specific virus amplification [14]. Transgene transfer by replication-incompetent virus has been less efficacious than hoped in clinical application due to limited infection and amplification. Replication-competent viruses have drawn widespread attention because viral replication within cancer cells results in oncolysis and produces progeny virions that can infect adjacent cells. Several studies have reported on conditionally replicating virus in prostate cancer using prostate-specific enhancer/promoter. Enhancer/promoters of human glandular kallikrein and prostate-specific-antigen (PSA) have been used to drive the E1A

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and/or E1B gene in PSA-positive prostate cancer cells [15,16]. The genetic element from the rat probasin promoter has also been inserted into Ad5 virus to control E1 genes together with the PSA enhancer/promoter [17]. However, these promoter/enhancers are likely to be less active in patients undergoing androgen ablation therapy because their actions are dependent on androgen.

Prostate-specific membrane antigen (PSMA) is a type II membrane protein that is expressed predominantly in the prostate epithelium. PSMA expression in prostate cancer is significantly higher than in benign prostate hyperplasia or the normal prostate and is greater in prostate cancer with a higher Gleason score [18–20], suggesting that the regulatory elements controlling PSMA expression become more active as prostate cancer progresses. There are at least three PSMA variants produced by alternative splicing: PSM′, PSM-C, and PSM-D. Interestingly, the ratio of full-length PSMA to variant PSM′, which lacks nucleotides 114 to 380 of the PSMA cDNA and, as a result, is located in cytoplasm, increases as normal prostate progresses to the tumor [20,21].

To date, two transcription regulatory elements have been characterized: the 1.2-kb promoter upstream of the PSMA-encoding gene (*FOLH1*) and the PSMA enhancer core (PSME) in the third intron of *FOLH1*. PSME exhibits high activity only in PSMA-positive LNCaP, C4-2, CWR22rv, and MDA Pca 2b cells; very low activity in PC-3 cells; and no activity in other PSMA-negative cells tested [22,23]. Consistent with PSMA expression, PSME is also negatively regulated by androgens, so it exhibits a much higher activity at low levels or in the absence of androgens [18,22,23], making PSME a strong candidate for mediating virus replication or expressing exogenous cytotoxic genes in hormone-refractory prostate cancer. Previously, PSME was used for the expression of the suicide gene cytosine deaminase [9] and the PSME/probasin promoter for the expression of a different cytotoxic gene, purine nucleoside phosphorylase [24]. In this study, we examined whether PSME-mediated replication of adenovirus can be used to treat prostate tumors in a low-androgen environment.

RESULTS

PSME Has Transcription-Enhancing Activity Only in PSMA-Positive Cells

PSME, located in the third intron of the PSMA-encoding *FOLH1* gene, confers the prostate-specific expression of PSMA and is responsible for the up-regulation of PSMA expression in the absence of androgen [22]. These properties make PSME a potential transcription regulatory element utilized exclusively in the prostate, specifically in an androgen-depleted environment. Prior to using PSME for the prostate-specific expression of exogenous genes in prostate, we constructed a luciferase reporter (pGL3/TATA/luciferase) controlled by PSME/TATA and evaluated its activity in PSMA-positive and negative cancer cells (Fig. 1).

We measured PSME enhancing activity in different cell lines by transient transfection and luciferase reporter assay as described under Materials and Methods. As shown in Fig. 1B, PSME drove a high level of luciferase expression in PSMA-positive prostate cancer cells LNCaP, C4-2, and CWR22rv. Its enhancing activity was up-

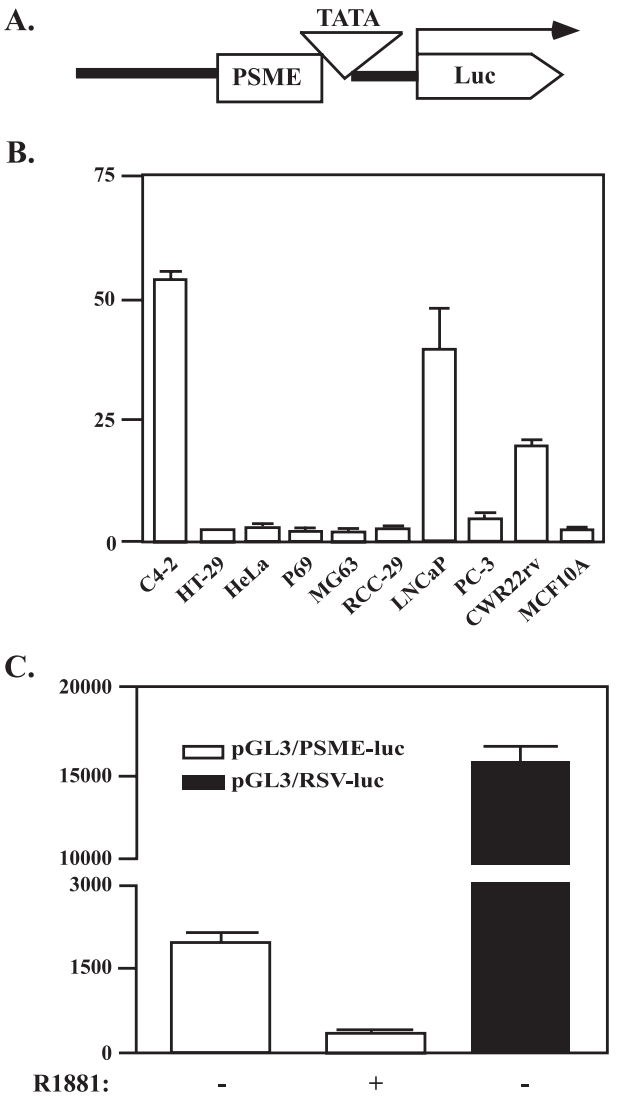

FIG. 1. The transcription enhancing activity of PSME. (A) PSME sequence was inserted upstream of TATA in pGL3/TATA. (B) The transcriptional activities of this construct were evaluated in the absence of androgen by transient reporter transfection assay as described under Materials and Methods. All data were normalized by cotransfection of 1 ng of a *Renilla* luciferase vector, pRL-SV40 (Promega). Results are presented as means ± standard error of three transfections. (C) The luciferase activity of pGL3/PSME/TATA or pGL3/RSV was measured in C4-2 cells in the absence of androgen. Relative luminescence is expressed as the mean ± standard error of the mean of at least three independent experiments.

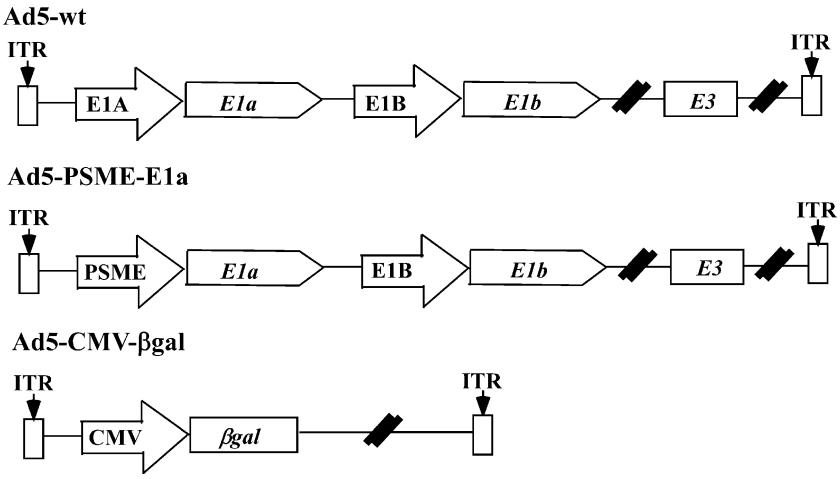

FIG. 2. The schematic organizations of the recombinant adenoviruses used in this study. PSME controls the expression of E1A, resulting in the selective replication of adenovirus Ad5-PSME-E1a in PSME-positive cells. Ad5-CMV-βgal is a replication-deficient virus due to deletion of the E1 and E3 regions. ITR, inverted terminal repeat.

regulated in the absence of androgen (Fig. 1C) and this androgen-mediated modulation of PSME activity correlates well with PSMA expression. In contrast, its activity in nonprostate cancer cells and PSMA-negative prostate cancer cells showed only a basal level of luciferase activity, supporting a previous report [22] on PSME function in PSMA-positive prostate cancer cells.

Recombinant Ad5-PSME-E1a Mediated High E1A Protein Expression in PSMA-Positive Prostate Cancer Cells

Since PSA-driven oncolytic adenovirus was introduced [16], several promoter/enhancers specifically active in prostate have been described. Most of them are, however, dependent on androgen receptor/androgen complex for action, so their activities would be diminished in prostate cancer patients after castration. Unlike other androgen-dependent promoters/enhancers, PSME activity increases almost threefold with androgen depletion, as shown in Fig. 1C. Next, we evaluated whether PSME could be used as a regulatory element to drive viral replication and kill prostate tumors at the low levels of androgen seen after androgen ablation therapy. We constructed and produced a conditionally replicating adenovirus (CRAD), Ad5-PSME-E1a, and two other viruses for these studies as described under Materials and Methods. These viral constructions are schematically depicted in Fig. 2. Unlike wild-type adenovirus, PSME in Ad5-PSME-E1a controls viral protein E1A expression and determines viral replication. In Ad5-CMV-βgal (β-galactosidase), βgal is substituted for E1 and E3 under the control of the cytomegalovirus (CMV) promoter as described in our previous report [23].

We tested whether PSME drives E1A expression and viral replication in different cell lines. We included Ad5-wt as a positive control and Ad5-CMV-βgal as a negative control in which E1 and E3 were deleted. We prepared lysates from virus-infected cells and then determined E1A expression by Western blot probed with antibodies reactive to E1A proteins (Fig. 3). PSMA-positive C4-2 and CWR22rv synthesized several proteins of 30–55 kDa translated from 9S, 10S, 11S, 12S, and 13S E1A transcripts, respectively. In contrast, the PSMA-negative prostate cancer cell PC-3 and lung carcinoma A549 showed the basal or lowest level of E1A expression. As expected, wild-type adenovirus infection resulted in a significant

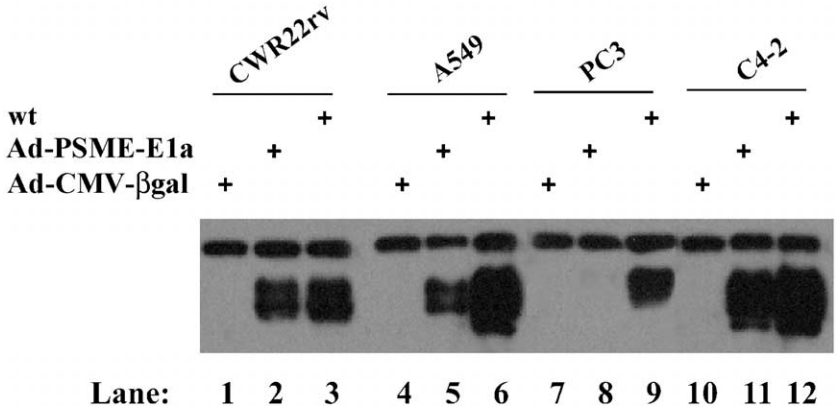

FIG. 3. E1A protein expression mediated by Ad-PSME-E1a. Androgen-independent prostate cancer cells were harvested 2 days after infection and lysed. Cell lysates were resolved by SDS-PAGE and transferred to PVDF membrane. E1A proteins were detected by Western blotting as described under Materials and Methods.

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amount of E1A proteins in all cell lines, while no E1A proteins were detected from cells infected with Ad5-CMV-βgal as a negative control. These experimental results demonstrated that, like PSME activity, PSME in the adenoviral genome mediated high E1A protein expression in PSMA-positive prostate cancer cells, CWR22rv and C4-2. Furthermore, we reasoned that Ad5-PSME-E1a replication would actively proceed only in PSMA-positive prostate cancer cells.

Infectious Virus Particle Production by Ad-PSME-E1a

To address whether PSME-mediated E1A expression could lead to high viral replication in PSMA-positive prostate cancer, we investigated the replication efficiency of Ad5-PSME-E1a and Ad5-wt in several different cell lines. Two days after infection with Ad5-PSME-E1a virus, we harvested cells and supernatant together and released virus particles by three freeze–thaw cycles. We titered the collected viral particles by plaque assay in 293 cells and normalized them to wild-type adenovirus replication. As shown in Fig. 4, Ad5-PSME-E1a replication in PC3 and A549 cells was 2 to 4 log orders less than in C4-2 and CWR22rv cells. Furthermore, androgen down-regulated Ad5-PSME-E1a replication in C4-2 and CWR22rv, consistent with PSME activity as shown in previous reports [22,23].

Cytopathic Effect of Conditionally Replicating Ad-PSME-E1a

We next tested the ability of AdPSME-E1a virus to induce cytopathic effects in tissue-cultured cancer cells (Fig. 5). We plated cells in six-well dishes and infected them with different numbers of virus particles. We counted surviving cells after 5 days by trypan blue staining. Consistent with the promoter activity and number of produced viral replications, C4-2 cells showed almost total cell kill by 0.1, 1, and 5 pfu (plaque-forming units) after 5 days. The total cell kill by Ad5-PSME-E1a was almost comparable to that of the wild-type adenovirus. More than 1 pfu of Ad5-PSME-E1a also elicited a total cell killing effect on CWR22rv. Ad5-PSME-E1a induced a low level of cytotoxicity in A549 lung cancer cells; however, similar toxicity was observed with Ad5-CMV-βgal, suggesting that the toxicity was nonspecific and caused by viral infection

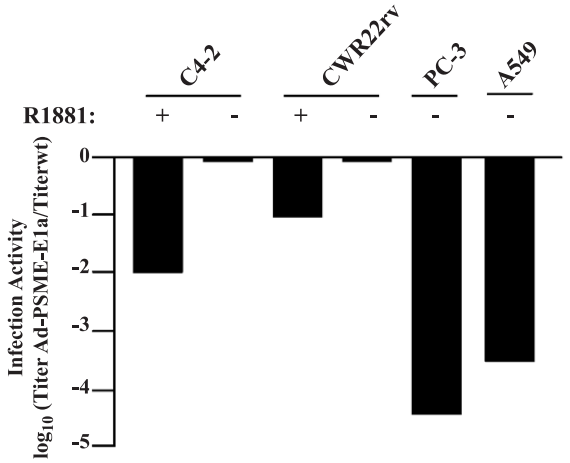

FIG. 4. Replication of Ad-PSME-E1a virus in several human cancer cells *in vitro*. Cells (1×10^6) were infected with 5 pfu of wild-type or Ad-PSME-E1a per cell. Viruses recovered from cells and media 2 days later were titered on confluent layers of 293 cells. Results are presented by $\log_{10}$ (titer$^{\text{Ad-PSME-E1a}}$/titer$^{\text{wt}}$).

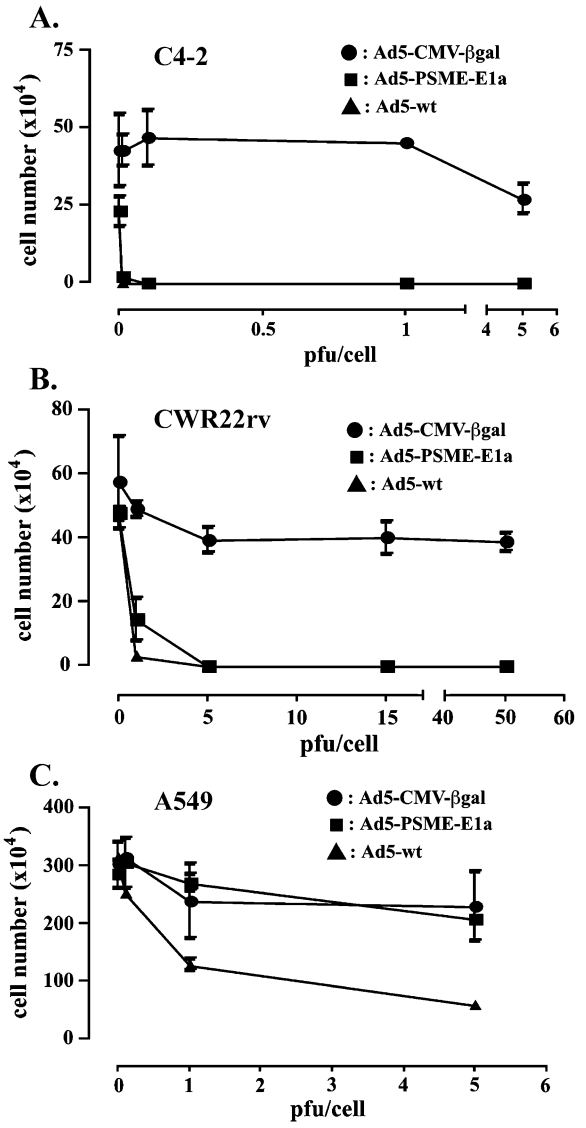

FIG. 5. Cytotoxicity efficacy of Ad-PSME-E1a on prostate cancer and A549 cells *in vitro*. Cells (5×10^4) were seeded onto six-well plates and infected with different numbers of pfu per cell as shown. After 5 days, viable cells were counted after staining with trypan blue. Each experiment was carried out in triplicate. The results showed that Ad5-PSME-E1a was cytotoxic to androgen-independent prostate cancer cells (C4-2 and CWR22rv).

itself. As shown in Fig. 5, Ad5-PSME-E1a was more cytopathic to C4-2 than to CWR22rv when both were infected with the same number of virus particles. Even though virus production does not necessarily correlate with the magnitude of the cytopathic effect [25], the greater effect on C4-2 could be attributed to the greater activity of PSME (see Fig. 1) and high expression of E1A protein in C4-2 cells (see Fig. 3). Although AR expression is greater in C4-2, compared with CWR22rv (data not shown), AR mutant (H874Y) in CWR22 and wild-type AR show higher activity in the presence of dihydrotestosterone at a near-physiological concentration, compared with AR mutant (T877A) in LNCaP [26]. As a result, it is difficult to use the AR to explain the PSME activity differences between CWR22rv and C4-2. Alternatively, C4-2 presumably has the more abundant expression of transcription factors involved in the regulation of PSME activity, such as NFATc1, SOX, and AP-1 [20,27].

In summary, PSME, whose activity is up-regulated in the absence of androgen, enabled E1A expression and viral replication and showed profound cell killing ability in tissue cultures at low androgen levels. In the following experiments, we investigated androgen-independent prostate tumor regression in an animal model using Ad5-PSME-E1a virus.

Tumor Regression Induced by Ad-PSME-E1a Injection

We examined the oncolytic efficacy of Ad5-PSME-E1a in tumors induced in mice. We established prostate tumors with CWR22rv prostate cancer cells in nude mice castrated to produce a low-androgen environment. When tumors were palpable, we subjected them to intratumoral injection of Ad5-PSME-E1a or Ad5-CMV-βgal. Subsequently we monitored the tumor burden once every week. As shown in Fig. 6A, Ad5-PSME-E1a induced marked tumor regression ($P < 0.05$). Three of eight tumors were completely eliminated and one of four mice was visually free of tumors. However, mice injected with Ad5-CMV-βgal failed to exhibit a decrease in tumor burden. These data, like the data above, suggest that PSME is active in CWR22rv tumor. Histological examination confirmed the effect of Ad5-PSME-E1a injection (Fig. 6B).

Tumors treated with Ad5-CMV-βgal were still growing healthily at the time of harvest, but tumors treated with Ad5-PSMA-E1a showed a substantial amount of dead cells (approximately 60–80% area in several slides) caused by the virus replication (Fig. 6B). As expected from CRAD, a number of adenovirus particles were detected in residual tumors in Ad5-PSME-E1a-treated animals as seen in the immunohistochemical staining analysis using antibodies reactive to adenovirus, but were barely detectable in Ad5-CMV-βgal-treated animals 28 days after the virus injection (Fig. 6C). These results suggest that extensive dead cells in the Ad5-PSME-E1a group were due to replicative virus production.

Discussion

Adenovirus has shown promise as an adjuvant agent for tumor eradication in prostate cancer patients. However, the limited infectivity of replication-defective adenovirus has resulted in poor clinical outcomes. Several approaches to increase virus replication exclusively inside tumor cells without damaging normal cells have been reported. Recent years have witnessed the development of replication-competent "oncolytic" adenoviruses, starting with the ONYX-015 virus, which was used to target p53-deficient cells since its deletion in the E1B 55-kDa gene resulted in its inability to replicate in normal cells [28]. Tumor-specific replication of adenovirus can also be achieved by using the regulatory element of telomerase reverse transcriptase (TERT), which is active in most cancer cells, to control the expression of adenovirus E1A genes. Alternatively, different approaches to enable the tissue-specific replication of adenovirus have used tissue-specific promoters like PSA [15], kallikrein 2 [29], and rat probasin [29] for prostate cancer and AFP for hepatocellular carcinoma. Such therapeutic approaches, along with more knowledge about viral protein functions, have contributed to the great progress that has been made in using adenovirus to treat various tumors, including prostate cancer.

We used the regulatory element (PSME) for prostate-specific membrane antigen expression to construct a prostate/prostate tumor-restricted replicative adenovirus. PSMA is up-regulated in most patients after hormone ablation therapy [18,22] and the up-regulation corresponds with tumor grade. This clinical association makes PSME a good regulatory element to replace an endogenous E1A promoter, enabling virus replication in hormone-refractory prostate cancer cells in patients undergoing androgen ablation therapy. In general, people believe that reactivation of the AR, either by ligand-independent or AR gene alterations (amplification or mutation), associates with androgen-independent progression of the prostate cancer disease[30]. However, AR activity is not fully functional at this stage, compared with that at physiological androgen concentration. Due to this property, PSME has several advantages over PSA [15] or PSE [31] promoters previously used for oncolytic adenovirus construction.

We confirmed the difference in selectivity of Ad5-PSME-E1a between PSMA-positive prostate cancer cells and PSMA-negative cells by progeny production assay. Since different cell lines show variable susceptibility to adenovirus, virus production was normalized by comparison with a nonselective wild-type adenovirus. As summarized in Fig. 4, the recombinant adenovirus Ad5-PSME-E1a replicated poorly in PSMA-negative prostate

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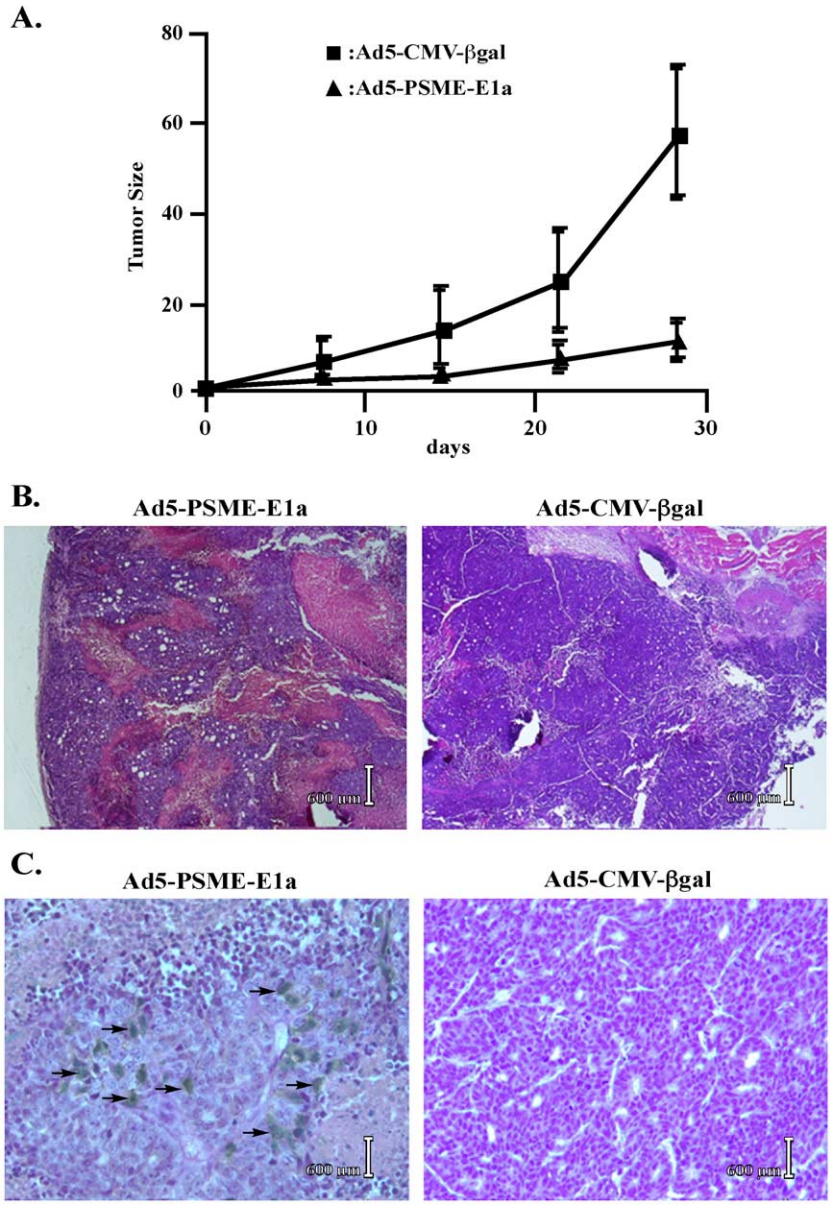

FIG. 6. Treatment of tumor xenografts with recombinant adenoviruses. Tumor xenografts (CWR22rv) were grown sc in athymic nude mice. (A) Tumor size was measured at different time points as indicated after intratumoral injection of Ad5-PSME-E1a (1.7×10^8 pfu) or Ad5-CMV-βgal. Each group included four mice with two tumors per mouse. (B) H&E staining of tumors obtained from mice treated with either Ad5-PSME-E1a or Ad5-CMV-βgal. Original magnification ×20. (C) Immunohistochemical staining with anti-adenovirus antibodies and counterstaining (H&E) were performed. Black arrows indicate stained adenovirus foci. Original magnification ×200.

cancer cells like PC3 and A549. Furthermore, as expected from the down-regulation of PSME activity in the presence of androgen, androgen induced less virus replication in the C4-2 and CWR22rv cell lines. Western blotting (Fig. 3) with anti-E1A antibodies as probes demonstrated that E1A protein expression correlated with the results from the progeny assay shown in Fig. 4. Almost 70% of prostate cancer cells express PSMA antigen, and as a result this antigen has been used in several prostate cancer therapeutic approaches [32–37]. Current studies suggest that PSME-driven replication-competent adenovirus has the potential to be a new therapeutic modality for prostate cancer patients specifically after hormone ablation therapy. Furthermore, this adenovirus can be made more potent when it is equipped with cytotoxic genes like HSV-TK, cytosine deaminase, and TRAIL to potentiate its therapeutic efficacy. We expect future clinical tests to show that PSMA-negative prostate cancers could also be eliminated by adenoviruses replicating within neighboring abundant PSMA-positive cells and expressing cytotoxic genes.

In this study, we constructed an oncolytic adenovirus controlled by PSME and tested its cytotoxic efficacy at low levels of androgen. Our data show that PSME-mediated oncolytic adenovirus may be a

valuable treatment for prostate cancer patients after hormone therapy.

MATERIALS AND METHODS

Cells and cell culture. The LNCaP prostate cancer cell line was obtained from Dr. Leland Chung. CWR22rv was a gift from Dr. Liang Cheng. The rest of cell lines used in this study were purchased from ATCC (Manassas, VA, USA). All cell lines were maintained in RPMI 1640 supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). All reagents for cell culture were purchased from Invitrogen/GibCo (Carlsbad, CA, USA).

Evaluation of luciferase activity in different cells. Full-length PSME for transient transfection was obtained and cloned into pGL3TATA vector as described in our previous report [23]. The transfection assay was done as described previously [23].

For transient transfections, 1×10^5 cells/well were plated in 12-well plates for 24 h. Plasmid pGL3/PSME/TATA or pGL3/TATA (0.5 μg) was transfected with 1 ng of *Renilla* luciferase pRL-SV40 (Promega, Madison, WI, USA) using DOTAP (Roche, Indianapolis, IN, USA) or Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. After transfection, cells were maintained in phenol red-free RPMI 1640 medium containing 5% charcoal-stripped serum and 1% P/S. After 48 h, cells were harvested and lysed in 250 μl passive lysis buffer (Promega). For luciferase activity detection, the total luminescence from 10 μl of the supernatant mixed with 50 μl of luciferase substrate (Promega) was measured by a femtometer (Zylux, Germany). Subsequently, 50 μl of stop solution (Promega) was added to the tube and mixed and *Renilla* luciferase activity was measured by a luminometer. Relative luminescence is expressed as the mean ± standard error of the mean of at least three independent experiments.

Construction of recombinant adenovirus. The adenovirus construction system was developed by Dr. Xavier Danthinne (O.D.260, Inc., Boise, ID). The system contains a cloning vector, pAd1020SfidA, containing the adenovirus left ITR and packaging signal (1 to 358 bp), and an adenoviral genome vector, pAd288, containing the right arm of adenovirus from 3504 bp to the end with E3. The PSME enhancer, E1A genes (with TATA box), and part of the E1B genes (to 3924) were cloned into pAd1020SfidA; then the left arm of the adenovirus was cloned into pAd288 via a three-way ligation, *Sfi*I–*Afl*II (from pAd1020SfidA cloning vectors), *Afl*II–*Pme*I, and *Pme*I–*Sfi*I (from pAd288). Lambda phage was applied to pack the ligation mix and to transduce bacteria, which were then plated onto an agar plate containing kanamycin. Plasmids were purified and digested with *Pac*I to release adenoviral genomes and transfected into PER.C6 cells using Lipofectamine2000 (Invitrogen) to generate recombinant adenoviruses. The media were aspirated and an agarose mixture (0.6% agarose with 10% FBS in MEM) was added to the cells 2 days after transfection. The plate was incubated at 37°C for 7–10 days after transfection to allow plaque formation. Plaques were isolated and resuspended in 200 μl phosphate-buffered saline (PBS) and used for amplification in PER.C6 cells.

Western blotting. Cells were infected with 5 pfu of Ad5-PSME-E1a and lysed with PBS supplemented with 1% SDS. Total proteins from infected cells were resolved by SDS–PAGE and transferred to PVDF membranes. After transfer, membranes were blocked using 5% nonfat dried milk in Tris-buffered-saline (TBS) (50 mM Tris–HCl and150 mM NaCl, pH 7.2) and incubated for 1 h at room temperature with 1:250 dilution of anti-E1A antibodies (0.2 μg/μl) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed three times with TBS supplemented with 0.05% Tween 20 and then incubated with a 1:2000 dilution of secondary antibodies (Pierce, Rockford, IL, USA) for 1 h at room temperature. After three washes with washing buffer, the bands were visualized using ECL detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Viral replication and cytotoxicity assays. Viral replication assays were performed as described elsewhere [28]. Briefly, 1×10^6 cells in the medium supplemented with or without synthetic androgen (R1881) were infected with 5 pfu of virus per cell for 2 h, at which time unabsorbed virus was removed by washing with a glycine–saline solution (pH 3.0). Forty hours after infection, the supernatant and cells were harvested, exposed to three freeze/thaw cycles to release virions, and titered on 293 cells. The results are the combination of three independent experiments. For the viral cytotoxicity assay, cells were plated onto six-well plates at 2×10^5 cells per well for 24 h. Virus was added, ranging from 0 to 1000 virus particles per cell as indicated in Fig. 5, and incubated for 5 days. The number of surviving cells was counted after trypan blue staining. Counting was performed in triplicate.

In vivo animal experiment, immunohistochemistry, and hematoxylin and eosin (H&E) staining. To demonstrate the oncolytic activity and tumor specificity of Ad5-PSME-E1a, two sites per athymic mouse (20–25 g) were inoculated sc with 4×10^6 cells suspended in 100 μl T medium containing 5% FBS. Testes were removed by orchidectomy to lower the serum androgen level 2 days after the injection of CWR22rv cells as described [38]. When tumors became palpable (4–5 mm in diameter), four animals were randomly assigned to two groups: group 1, Ad5-PSME-E1a; group 2, Ad5-CMV-βgal. A single dose of virus (1.7×10^8 pfu) was injected intratumorally into the mice. After administration of the test viruses, tumor sizes were measured weekly and calculated according to the formula length × width^2 × 0.5236. Unpaired *t* test was utilized to determine the significance of size difference between tumors treated with Ad5-PSME-E1a or with Ad5-CMV-βgal.

Tumors in the nude mice treated with either Ad5-PSME-E1a or Ad5-CMV-βgal were fixed with 10% formalin and embedded in paraffin. The embedded tumors were then cut, dewaxed with ethanol, and stained with H&E. Adenovirus particles in tumor sections were analyzed by immunohistochemistry using polyclonal antibodies against adenovirus (Abcam, Cambridge, MA, USA) and counterstained with H&E.

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