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PSA promoter-driven conditional replication-competent adenovirus for prostate cancer gene therapy

Research Article

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Abbreviations: β-galactosidase, (lacZ); adenovirus type 5, (Ad5); Dulbecco's modified Eagle medium, (D-MEM); early region 1, (E1); Fetal bovine serum, (FBS); prostate specific antigen, (PSA); Rous sarcoma virus, (RSV)

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Summary

A conditional, replication-competent adenovirus (AdPSAE1) carrying the adenoviral E1 region under the control of a prostate specific antigen (PSA) promoter was generated in an effect to target the prostate for cancer gene therapy. The anti-prostate tumor efficacy and specificity of AdPSAE1 were examined *in vitro* and *in vivo* in prostate and nonprostate cancer models. *In vitro* at multiplicity of infection (moi) of 1, AdPSAE1 effectively killed the human prostate cancer cell lines PPC-1 and LNCaP, but had no effect on nonprostate cancer cells including the human bladder cancer cell line RT4, human breast cancer cell line MCF-7, and rat gliosarcoma cell line 9L. As a control, an adenovirus expressing the ß-galactosidase transgene under the control of the same PSA promoter (AdPSAlacZ) was used in parallel in all experiments. The *in vivo* tissue-specific expression driven by this PSA promoter was examined in a xenograft tumor model. Intratumoral injection of AdPSAlacZ resulted in PSA promoter-driven expression of lacZ in xenograft tumors in nude mice derived from human prostate cancer PPC-1 cells, but not in tumors derived from human bladder cancer RT4 cells. Intratumoral injection of AdPSAE1 effectively inhibited *in vivo* growth (61.8% reduction in tumor size) of xenograft PPC-1 prostate tumors compared to untreated or AdPSAlacZ treated tumors. Conversely, intratumoral injection of AdPSAE1 had no effect on the growth of xenograft RT4 bladder tumors when compared to untreated control group. These results indicate that prostate-targeted conditional replication-competent adenoviruses may be useful in gene therapy of prostate cancer.

I. Introduction

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer deaths in men today. It is estimated that there will be approximately 230,110 new cases and 29,900 deaths of prostate cancer in American men in 2004 (Jemal et al, 2004). Unfortunately for those patients diagnosed with advanced prostate cancer, there is no effective current treatment modality and their prognosis is poor. Although viral based gene therapy is a promising new strategy to combat advanced prostate cancer, its current effectiveness is limited by inefficient cellular transduction *in vivo*.

The adenovirus early region 1 (E1) gene, which comprises E1a and E1b, encodes the viral early proteins that are necessary for adenoviral replication and the consequent oncolysis of permissive host cells. E1-deleted (including E1a-deleted) adenoviruses are replication-defective and are commonly used as viral vectors to carry therapeutic genes for gene therapy. The conventional way of producing an E1-deleted adenovirus is to use cells that are able to supply replication-enabling proteins. One such example is HEK 293 cells which were transformed by human adenovirus type 5 (Ad5) and express E1 protein (Graham et al, 1977). E1-deleted viruses infect host cells and express the transgene but they cannot replicate and undergo lysis due to the lack of the E1 protein. Thus, E1-deleted recombinant adenoviruses are a safe viral vehicle for gene transfer. However, E1-deleted, replication-defective adenoviruses have several common problems with respect to *in vivo* transduction: a low transduction rate, time-limited expression of the transgene, and host immune responses to repeated viral administration.

An alternative means of producing E1-deleted adenoviruses is to provide the E1 protein in the targeted cells. Codelivery of an E1-deleted adenovirus along with an E1-expressing plasmid allows one round of viral replication. This limited replication significantly increases in vivo delivery efficiency of adenovirus to cancer cells (Goldsmith et al, 1994; Han et al, 1998). This *trans* complementation of a replication-defective adenovirus with E1 protein in targeted cells may provide a means of amplifying gene transduction *in vivo*. However, the resultant adenovirus itself is not replication-competent and only one round of viral replication is possible. Therefore, transduction of tumor cells by this approach is still limited.

Replication-competent viruses, also known as oncolytic viruses, replicate within transduced cells and force these cells into a lytic cycle. Released virus is then able to infect neighboring cells until all susceptible cells are eliminated. Theoretically a large tumor burden could be effectively eradicated using a small dose of an oncolytic virus. Therefore, strategies to use conditional oncolytic virus, or so-called attenuated replication-competent viruses, to specifically target prostate tissue have been developed (Rodriguez et al, 1997; Yu et al, 1999a, 1999b).

The idea behind this study is to place the Ad5 E1 region in *cis* complementation (i.e., use E1 as a transgene) back into an E1-deleted, replication-defective adenovirus under the control of a prostate-specific promoter. Thus, E1 protein expression will be confined strictly to prostate tissues and render this a conditional oncolytic virus within the prostate. Our previous study showed that a prostate-specific adenovirus, AdPSAlacZ, which contains a ß-galactosidase (lacZ) reporter gene under the control of the PSA promoter, transduced a high level of lacZ transgene expression in the prostate after intraprostatic injection in an animal model. The virus did disseminate to tissues beyond the prostate after injection, however, AdPSAlacZ did not express the transgene in these nonprostate tissues (Steiner et al, 1999). This result suggests that the PSA promoter effectively and specifically drives lacZ transgene expression in prostate cells transduced by AdPSAlacZ. In this study we replaced the lacZ transgene in AdPSAlacZ with the Ad5 E1 region to generate a prostate-specific replication-competent adenovirus AdPSAE1, in which E1 expression is under the control of the PSA promoter. The efficacy and specificity of AdPSAE1 as a potential therapeutic vector for prostate cancer gene therapy were analyzed.

II. Materials and methods

A. Cell culture and medium

Dulbecco's modified Eagle medium (D-MEM) was purchased from Gibco BRL (Gaithersburg, MD). RPMI 1640 medium and McCoy's 5α medium were purchased from Cellgro (Herndon, VA). Fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT). All cell lines were purchased from ATCC (Rockville, MD) and were grown in D-MEM with 10% heat inactivated FBS. The human prostate cancer cell lines PPC-1 and LNCaP, both secret PSA (Dr. J. Norris of MUSC, personal communication), were grown in RPMI 1640 medium with 10% FBS. The human breast carcinoma MCF-7 cells and human bladder cancer RT4 cells were grown in McCoy's 5α medium with 10% FBS. Rat gliosarcoma 9L cells were grown in D-MEM medium with 10% FBS. All cells were grown in medium containing 100 units/ml penicillin, 100 µg/ml streptomycin at 37°C in a 5% CO_2 atmosphere.

B. Construction of adenoviral vector AdPSAlacZ and AdPSAE1

The generation of AdPSAlacZ, an E1-deleted recombinant adenovirus expressing the lacZ reporter gene under the control of a 680-bp PSA promoter, has been described previously (Steiner et al, 1999). AdPSAE1 was generated by replacing the lacZ transgene in AdPSAlacZ with the wild-type Ad5 E1 gene. Briefly, an approximately 3-kb E1 fragment was generated by PCR using DNA extracted from the E1-containing adenovirus Ad-dl327 (Genetic Therapy Inc., Gaithersburg, MD) as a template, and primers specific to both the 5' and 3' region of the Ad5 E1 gene. In addition, a restriction site was introduced in each of the 5' and 3' primers to facilitate subsequent subcloning. The resultant PCR product included 4 bp upstream of the E1a gene start codon, the entire E1a and E1b regions, and 7 bp downstream of E1b stop codon, as well as the introduced BamH I and EcoR I site at 5'- and 3'- end, respectively. This PCR product was digested with BamH I and EcoR I, and subcloned into the corresponding sites in pBluescript (Stratagene, La Jolla, CA) and the E1 fragment was re-released with Spe I and EcoR V digestions. The prostate-specific adenoviral shuttle vector pPSAlacZ (used to generate AdPSAlacZ, Steiner et al, 1999) was digested with Xba I and Cel II to remove the lacZ gene, and was then ligated with the above-mentioned modified E1 fragment to generate the shuttle vector pPSAE1. This pPSAE1 shuttle vector was cotransfected with pJM17, an adenoviral genome plasmid, in 293 cells as described previously (Steiner et al, 2000a) to generate AdPSAE1. The resultant AdPSAE1 was genomically similar to Ad-dl327 except that the E1 gene in AdPSAE1 is under the control of a 680-bp PSA promoter rather the endogenous E1 promoter in Ad-dl327. Positive recombinant plaques were isolated by a direct plaque-screening PCR method (Lu et al, 1998) using primers specific to the recombinant construct, i.e., using one primer specific for the PSA promoter and the other primer specific for the E1 gene. Amplification and titration of adenoviruses were performed as described previously (Graham and Prevec, 1991).

C. Analysis of potential oncolytic effects of AdPSAE1 on various cell lines by crystal violet staining

Cells (5×10^4 per well) were plated in six-well plates, the next day the cells were either untreated or transduced with AdPSAlacZ or AdPSAE1 at moi of 1. After 6 days of transduction, the media was removed and the plates were washed twice with PBS. The wells were then completely covered with 2 ml of 1% crystal violet (Sigma, St. Louis, MO) and the plate was allowed to sit 5 min with gentle rocking. After washing with water, the plate was allowed to dry at room temperature overnight before they were photographed.

D. *In vitro* growth inhibition assay by AdPSAE1

Cells (5×10^4 per well) were plated in six-well plates, the next day the cells were divided into three groups: (a) control uninfected, (b) control virus AdPSAlacZ infected, and (c) AdPSAE1 infected. After viral infection at moi of 1, cell numbers were counted daily through day 6 post viral infection.

E. X-gal staining of AdPSAlacZ transduced xenograft tumors

The recombinant adenovirus, AdRSVlacZ, which contains a β-galactosidase reporter gene under the control of a Rous sarcoma virus (RSV) promoter, was used as a positive control to demonstrate *in vivo* transduction efficiency within tumors. Xenograft tumors were established by injecting 5 x 10^6 various cancer cells subcutaneously into the flank of male Balb/c nu/nu athymic nude mice (Harlan Sprague Dawley, Inc., Indianapolis, IN). When tumors reached about 50 mm^3 volume, 5 x 10^9 pfu AdRSVlacZ, or 1x10^{10} pfu AdPSAlacZ were injected directly into the tumor site. The mice were sacrificed 3 days post injection and the tumors were harvested and processed to cryosections as described previously (Lu et al, 1999). For tumor section staining, samples were fixed in 4% paraformaldehyde for 30 min, then in 30% sucrose in PBS at 4°C until the samples sank to the bottom of the vial. The samples were then snap-frozen in liquid nitrogen in O.C.T. medium (Tissue-Tek/Sakura, Torrance, CA) and processed to cryosections using a Cryostat. The cryosections were fixed in formalin for 30 sec then processed for X-gal staining as a measure of lacZ expression as described (Eastham et al, 1996).

F. *In vivo* tumor growth inhibition by AdPSAE1

PPC-1 cells (1×10^7 cells in 0.2 ml of PBS) or RT4 cells (5.7×10^6 cells in 0.2 ml of PBS) were injected subcutaneously into the flank of male Balb/c nu/nu athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN). For each tumor cell model, three groups of mice were formed with 8 mice in each group. Group I was used as an untreated control. Group II and group III were for intratumoral viral injection of AdPSAE1 and control virus AdPSAlacZ, respectively. When tumors reached about 200 mm^3 volume, a single dose of 5×10^6 pfu AdPSAE1 or AdPSAlacZ were injected directly into each tumor mass. Tumor volume was measured every 3 days until the animals were sacrificed. All of the animals were sacrificed at day 35 after viral injection, when several mice of group III showed distress or had tumor burdens > 15% of their total body weight.

III. Results

A prostate-specific, conditional oncolytic adenovirus, AdPSAE1, was generated by replacing the lacZ transgene of AdPSAlacZ (Steiner et al, 1999) with the wild-type Ad5 E1 region (**Figure 1**). This strategy allows the expression of E1 protein under the control of a prostate specific promoter (PSA), enabling the adenovirus to replicate and enter the oncolytic cycle only in prostate cells. To analyze the oncolytic cell-killing effects and tissue specificity of AdPSAE1, various cancer cell lines including prostate and nonprostate cells were used in both *in vitro* and *in vivo* models.

A. AdPSAE1 effectively and specifically inhibited prostate cancer cell growth *in vitro*

The potential oncolytic cell-killing effects of AdPSAE1 were analyzed in various cancer cells. The human prostate cancer lines PPC-1 and LNCaP and nonprostate cancer cell lines RT4 (human bladder cancer), MCF-7 (human breast cancer), and 9L (human glioma) were infected with AdPSAE1 or control virus AdPSAlacZ at moi of 1. Viable cells were stained with crystal violet 6 days after infection and were compared to untreated control cells (**Figure 2**). As dead cells typically detach, crystal violet stains only those viable cells that remain attached to the culture dish. As shown in **Figure 2A** and **2B**, AdPSAE1 (right well) almost completely wiped out all PPC-1 and LNCaP cells, whereas AdPSAlacZ (middle well) had no cell-killing effects as compared to the untreated control (left well), respectively. On the other hand, AdPSAE1 had no cell-killing effects on RT4 (**Figure 2C**), MCF-7 (**Figure 2D**) and 9 L (**Figure 2E**) cells. These results clearly demonstrate that AdPSAE1 selectively replicates (thus goes through the oncolytic cycle and kills the host cells) in cancer cells derived from the prostate (PPC-1 and LNCaP), but not in nonprostate cancer cells (RT4, MCF-7 and 9L).

To analyze the time-course of the growth inhibition effects of AdPSAE1 on prostate cancer cells, PPC-1 and LNCaP cells were either untreated or transduced with AdPSAE1 or control virus AdPSAlacZ at moi of 1 *in vitro*, and the cell numbers were monitored. As shown in **Figure 3**, significant growth inhibition was observed starting at day 4 post AdPSAE1 infection, with complete growth inhibition at day 6 for both prostate cancer cell

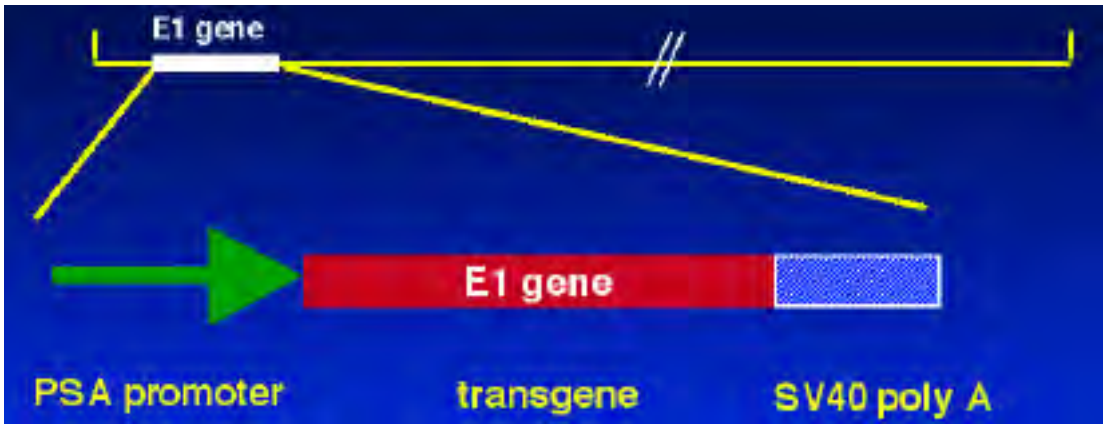

Figure 1. Design of a prostate-specific conditional replication-competent adenovirus. The native Ad5 early region 1 (E1) gene that is required for adenoviral replication, is replaced by an expression cassette which contains an Ad5 E1 gene under the control of a 860-bp PSA promoter.

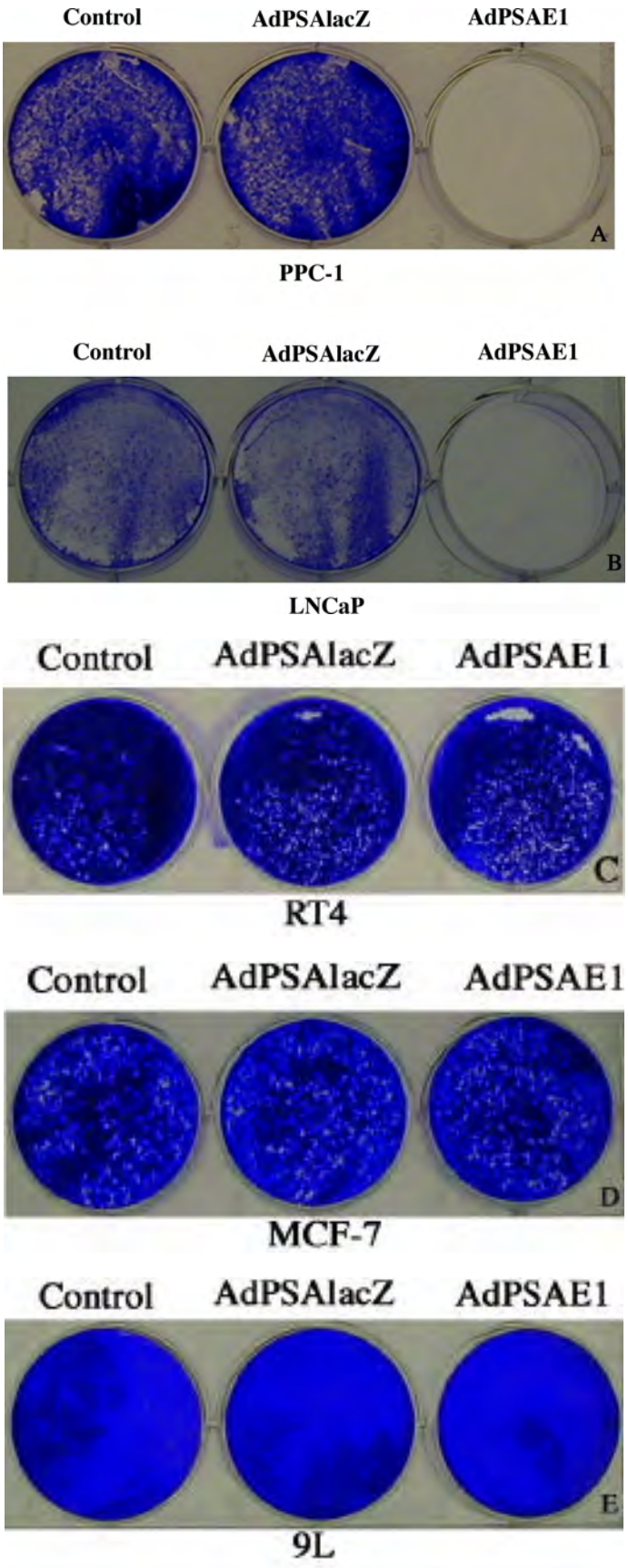

Figure 2. Conditional oncolytic effects of AdPSAE1 in prostate cancer cells. The human prostate cancer cell lines PPC-1 (**A**) and LNCaP (**B**), human bladder cancer cell line RT4 (**C**), human breast cancer cell line MCF-7 (**D**), and human glioma cell line 9L (**E**) were transduced with AdPSAE1 or AdPSAlacZ at moi of 1. Attached viable cells were stained with crystal violet 6 days after viral infection and were compared to the untreated controls.

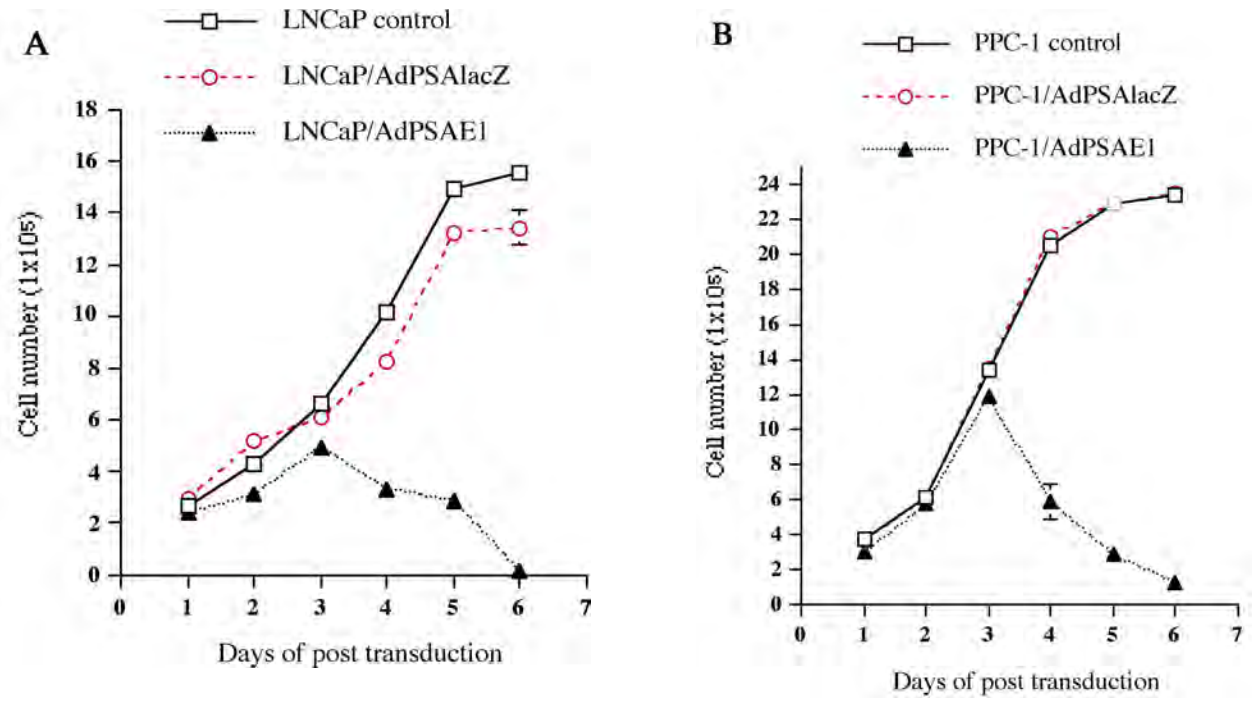

Figure 3. Time-course of the growth inhibition effects of AdPSAE1 on prostate cancer cells. Prostate cancer cells PPC-1 (**A**) and LNCaP (**B**) were transduced with AdPSAE1 at moi of 1. Cell numbers were determined daily from day 1 to 6 after viral transduction. Untreated and AdPSAlacZ transduced cells were used as controls. The data represent the results from two independent experiments each performed in duplicate. Some error bars are too small to show.

lines PPC-1 and LNCaP. AdPSAlacZ transduction did not cause significant growth inhibition in either of these cell lines (**Figure 3A** and **3B**).

The differential sensitivity of various cancer cells to AdPSAE1-mediated oncolytic killing and growth inhibition is presented in **Figure 4**. On day 6 after *in vitro* viral transduction at moi of 1, AdPSAE1 transduction significantly reduced numbers of PPC-1 and LNCaP cells to 81.6% and 96.9% of untreated control values, whereas the control virus AdPSAlacZ transduction resulted in minor and insignificant growth inhibition (**Figure 4**). In contrast, AdPSAE1 had no significant cell-killing or growth inhibition effects towards the nonprostate cancer cells RT4, MCF-7 and 9L when compared to the untreated control and control virus AdPSAlacZ transduced groups (**Figure 4**). These results suggest that, *in vitro*, AdPSAE1 effectively leads to prostate-specific oncolytic killing.

To ensure that selective viral replication accounted for the cell-killing in AdPSAE1 transduced cells, RT-PCR was performed using primers specific to Ad5 E1a gene and followed by Southern blot hybridization (Steiner et al, 1999) to examine the E1a mRNA expression in AdPSAE1-transduced cells. We found that only LNCaP and PPC-1 cells had positive E1a RT-PCR product whereas RT4, MCF-7 and 9L cells did not (not shown), indicating that E1a was selectively expressed in prostate cancer cells. We also performed RCA (replication complement adenovirus) assay by sequential infection of target cells (prostate and nonprostate cells) with AdPSAE1 and consequently collected supernatant of target cells to infect 293 cells. We only found plaques in 293 cells infected by supernatant from LNCaP and DU145 cells that

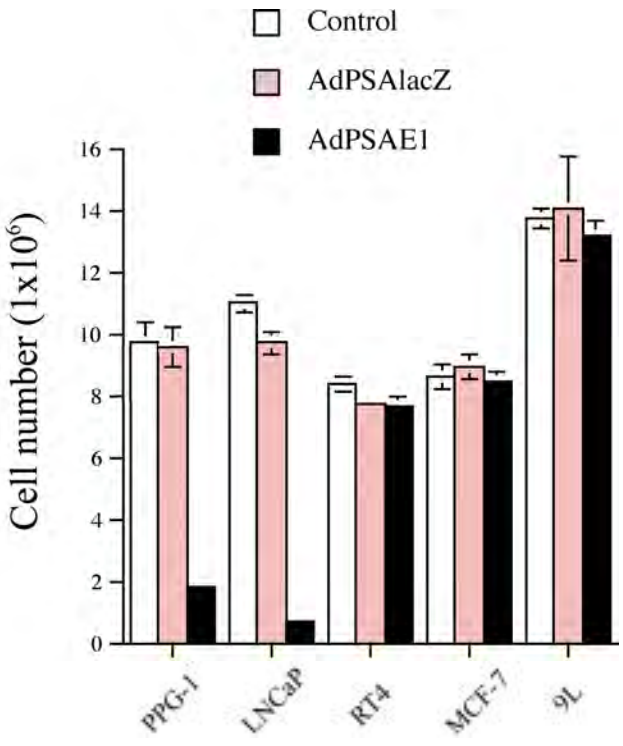

Figure 4. Differential growth inhibition of AdPSAE1 with respect to prostate and nonprostate cancer cells. Prostate cancer cells (PPC-1 and LNCaP) and nonprostate cancer cells (RT4, MCF-7 and 9L) were transduced with AdPSAE1 or AdPSAlacZ at moi of 1. Cell numbers were determined six days later and compared to that of untreated control. The data represent the results from two independent experiments each performed in duplicate. Some error bars are too small to show.

had been initially infected by AdPSAE1, not by supernatant from nonprostate cancer cells infected by AdPSAE1 (not shown). These results indicate that only AdPSAE1-transduced prostate cancer cells generate progeny viruses.

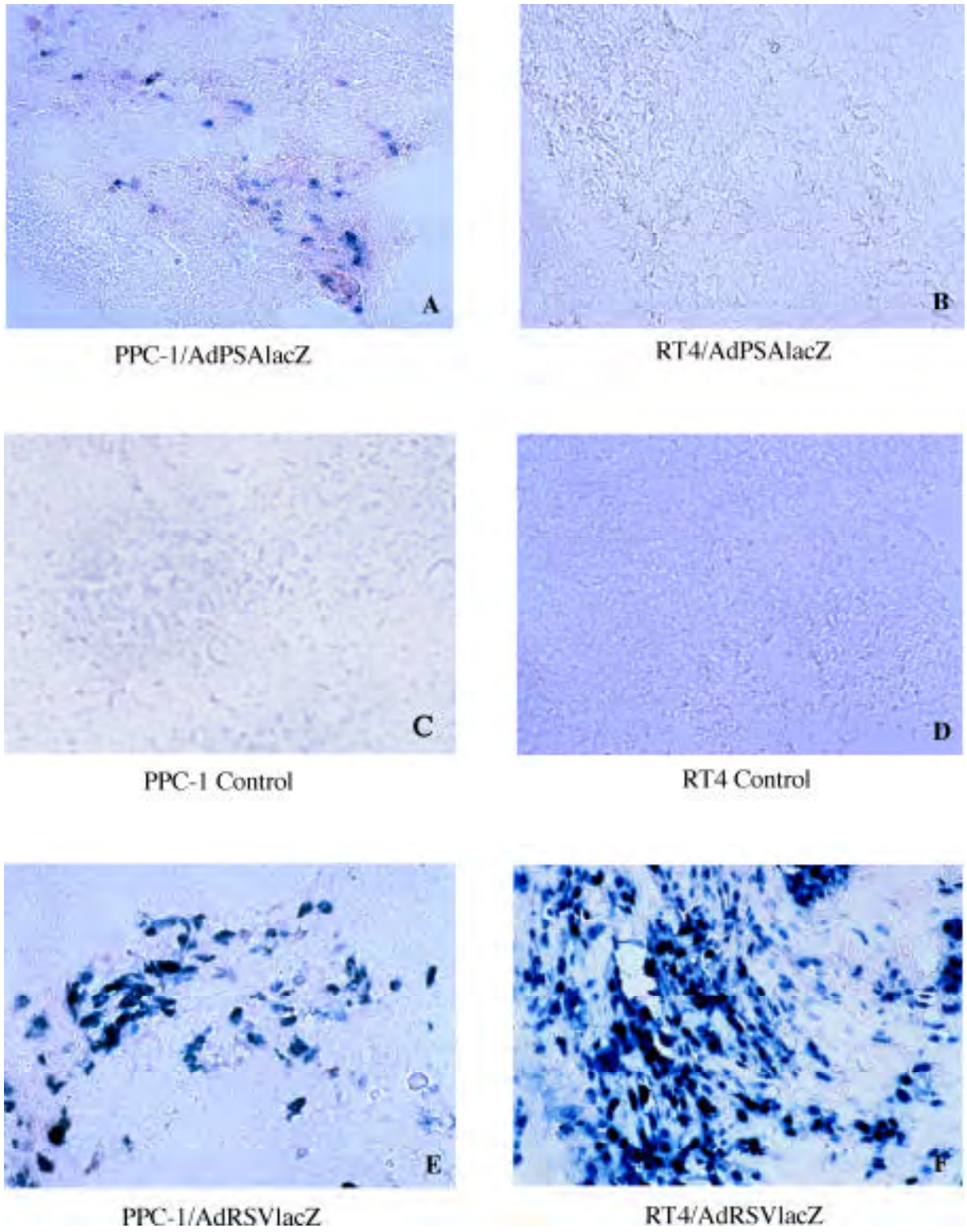

Figure 5. Specific transgene expression driven by a PSA promoter in prostate cancer cells. Xenograft tumors were established by subcutaneous injection of cancer cells into the flank of nude mice. When tumors reached about 50 mm^3, each of the adenoviral constructs ($1x10^{10}$ pfu AdPSAlacZ or $5x10^9$ pfu AdRSVlacZ) was injected directly into the tumor. The tumors were harvested 72 hr later and processed to cryosections. Shown are X-gal staining of tumor sections derived from prostate cancer PPC-1 cells (**A, C, E**) and bladder cancer RT4 cells (**B, D, F**). A and B are tumors transduced by AdPSAlacZ ($1x10^{10}$ pfu). C and D are untreated control tumors to serve as negative controls. E and F are tumors transduced by AdRSVlacZ ($5x10^9$ pfu) to serve as positive controls.

B. Specific expression of transgene driven by the PSA promoter in the xenograft prostate tumors in animal model

To determine the *in vivo* specificity of a 680-bp PSA promoter that was used tin the AdPSAE1 construct, a parallel adenovirus, AdPSAlacZ, containing a lacZ reporter gene under the control of the same 680-bp PSA promoter was used to analyze specificity in xenograft tumors grown in nude mice. A dose of $1x10^{10}$ pfu AdPSAlacZ was injected into subcutaneous xenograft tumors derived from human prostate cancer PPC-1 cells or human bladder cancer RT4 cells. As a positive control, AdRSVlacZ (Lu et al, 1999), an adenovirus containing the lacZ gene under the control of a constitutively active RSV promoter, was injected into xenograft tumors at a dose of $5x10^{9}$ pfu. LacZ expression was determined through X-gal staining of cryosections of the tumors 72 h following viral injection. Untransduced control PPC-1 (**Figure 5C**) and RT4 (**Figure 5D**) tumors did not express detectable endogenous lacZ. AdPSAlacZ transduced PPC-1 tumors contained X-gal positive (blue stained) cells (**Figure 5A**), whereas AdPSAlacZ transduced RT4 tumors did not (**Figure 5B**). In contrast, both PPC-1 (**Figure 5E**) and RT4 (**Figure 5F**) tumors transduced by AdRSVlacZ showed X-gal positive cells. These results demonstrate that expression of the lacZ transgene driven by this PSA promoter occurred only in xenograft prostate tumors, but not in xenograft bladder tumors. However, the activity of the PSA promoter is much lower than that of the constitutively active RSV promoter (Compare **Figure 5A** and **5E** with the blue stained cells and the viral dose injected, respectively).

C. dPSAE1 specifically inhibited prostate tumor growth *in vivo*

To determine whether AdPSAE1 causes similar tumor growth inhibition *in vivo* as was shown *in vitro* (**Figure 2, 3** and **4**), human prostate cancer PPC-1 cells and human bladder cancer RT4 cells were injected subcutaneously into the flank of nude mice to establish the xenograft tumors. When tumors developed to about 200 mm^3, a single dose of AdPSAE1 was injected directly into the tumor in both cancer cell models. As shown in **Figure 6A** for the PPC-1 tumor model, both untreated tumors and tumors treated with control virus AdPSAlacZ grew rapidly and at a similar rate. In contrast, the AdPSAE1-treated group showed an effective suppression of this rapid growth. By day 35 post viral injection, the group treated with AdPSAE1 had a remarkable 61.8% reduction of tumor size as compared to the untreated group (**Figure 6A**). On the other hand, the same single dose of AdPSAE1 injected into the RT4 xenograft tumors failed to result in significant growth inhibition, as compared to the untreated RT4 tumor group (**Figure 6B**). These results suggest that AdPSAE1 is able to specifically inhibit prostate tumor growth *in vivo*.

IV. Discussion

Most currently used gene therapy vectors are engineered to prevent viral self-replication. These replication deficient viruses represent a safer gene transfer vehicle. They deliver therapeutic transgenes without exposing host cells to the viral lytic cycle. The transduction of replication-defective viral vectors *in vivo* confines transgene expression to those cells along the

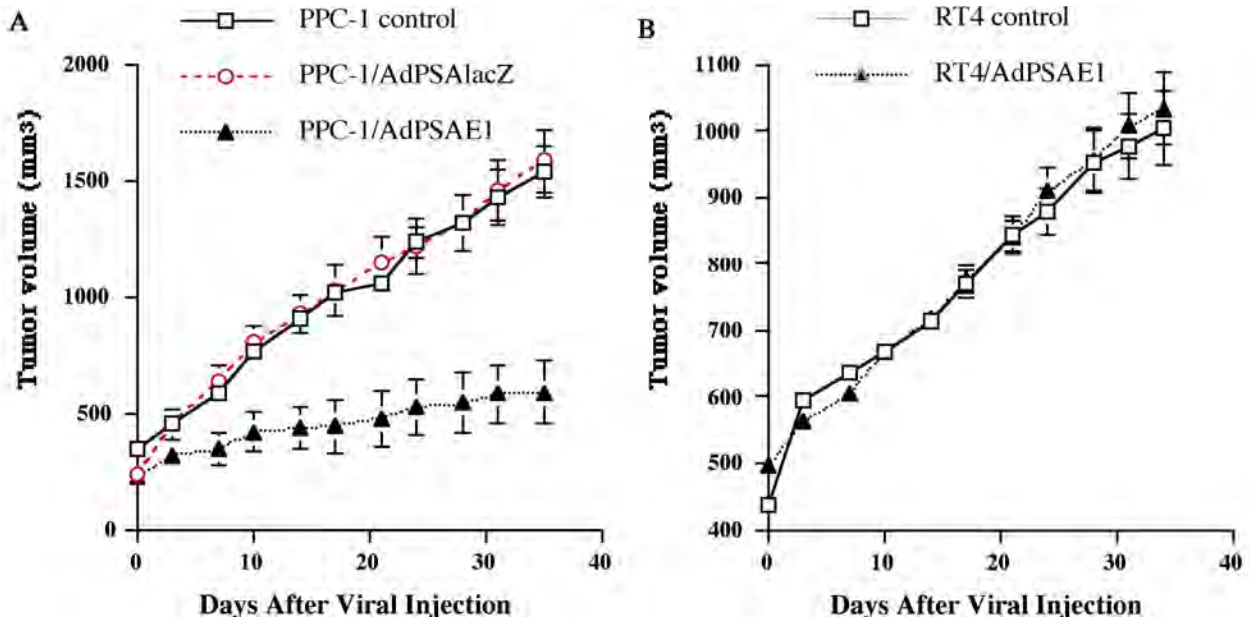

Figure 6. AdPSAE1 specifically inhibits prostate tumor growth *in vivo*. (**A**) The human prostate cancer line PPC-1 and (**B**) human bladder cancer line RT4 were injected subcutaneously into the flank of nude mice. When tumors reached an average volume of 200 mm^3, tumors were either untreated (control) or treated with intratumoral injection (day 0) with $5x10^{6}$ pfu of AdPSAlacZ (control virus) or $5x10^{6}$ pfu AdPSAE1. The tumor sizes were periodically measured after viral injection. Each point represents the average tumor volume from 8 mice. Some error bars are too small to show.

injection track due an inability to pass the transgene to neighboring cells. Consequently, the effectiveness of a viral vector is directly correlated to its transduction efficiency. Although bystander effect of certain therapeutic transgenes in the suicide gene therapy strategy helps to increase some therapeutic index, its effect is limited. Tumor cells cannot be 100% transduced with a single treatment. Untransduced tumor cells survive, divide and eventually offset the therapeutic effects posed by the initial viral transduction. Therefore, repeated viral injections aimed at infecting those tumor cells not infected in the first round of viral transduction is required to maximize the therapeutic effect *in vivo*. However, adenoviral vectors cause strong immunogenic responses. Consequently, second and subsequent rounds of adenoviral administration possess significantly reduced therapeutic effects *in vivo* (Berkner, 1988; Russell, 2000).

To overcome this obstacle, an alternative approach is to employ conditional oncolytic viruses, also called attenuated replication-competent viruses, for cancer gene therapy. Conditional oncolytic viruses are altered such that they specifically target a desired cell type or modified such that the desired target cells are several orders of magnitude more sensitive to oncolytic cell lysis than are nontargeted cells. By taking advantage of prostate-specific promoter, an Ad5 E1a gene, was reintroduced to E1a/E3-deleted adenovirus under the control of PSA enhancer/promoter (-5322 to –3729/-580 to +12) (PSE). The resultant adenovirus, CN706, specifically replicates in, and thus kills, PSA-producing cells such as LNCaP but not in non-PSA-producing cells such as DU145 (Rodriguez et al, 1997). Likewise, CN764, an adenoviral vector containing the Ad5 E1a gene driven by PSE and the Ad5 E1b gene driven by a hK2 enhancer/promoter (-5155 to –3387/-324 to +33), has a high therapeutic index with a cell specificity of 10,000:1 for prostate cancer LNCaP cells, compared to ovarian cancer OVCAR-3, SK-OV-3 and PA-1 cells (Yu et al, 1999a). A similar approach was used to generate another prostate-specific replication-competent adenovirus, CV787. CV787 contains the E1a transgene driven by a prostate-specific probasin promoter, an E1b gene driven by the PSE promoter and a wild-type E3 region that suppresses the host immune system. CV787 destroys PSA-producing cells 10,000 times more efficiently than non-PSA-producing cells. A single tail vein injection of CV787 has been shown to eliminate distant LNCaP xenograft tumors (Yu et al, 1999b). This indicates that CV787 could be a powerful therapeutic vector to treat metastatic prostate cancer.

Unlike other groups as mentioned above in which they used much longer PSA promoter region (above 1.6 kb), our current study shows that a 680-bp PSA promoter is sufficient enough to drive a prostate-specific transgene expression. This 680-bp PSA promoter drives expression of the lacZ transgene specifically in xenograft tumors derived from prostate but not in those derived from nonprostate cancer cells (**Figure 5A** and **5B**). This demonstrates specific expression of transgene by the PSA promoter only in prostate derived cells. Our previous publication demonstrated that the same PSA promoter drives expression of the reporter transgene in a prostate-specific manner when AdPSAlacZ was directly injected into the prostate (Steiner et al, 1999). The majority of injected virus was retained within the prostate gland, whereas a minor portion spread to distant tissues. Despite nonprostate infection by the adenovirus as detected by Southern blot of PCR using primers specific to the Ad5 adenovirus, the lacZ transgene was not expressed as detected by Southern blot of RT-PCR using primers specific to bacterial lacZ gene. Together, these data strongly demonstrate that the 680-bp PSA promoter drives transgene expression exclusively in the prostate *in vivo*.

In this study we used xenograft prostate tumors derived from a primary prostate cancer cell line PPC-1 (Brothman et al, 1989), rather from a metastatic prostate cancer line (such as LNCaP or DU145 as other groups did), for analyzing the efficacy of AdPSAE1. We believe that the intratumoral injection of viral vector into a primary prostate tumor setting reflects much closer to the real clinical situation for prostate cancer gene therapy. Moreover, to our knowledge, we are the first group to use a bladder xenograft tumor model (RT4) for analyzing the specificity of PSA promoter-driven E1 expression (**Figure 6B**), it seems to make more sense to us to pay attention whether AdPSAE1 would cause damage to the bladder, which is anatomically close to the prostate during the prostate cancer gene therapy application, rather than to the ovarian and breast as used by other group (Yu et al, 1999a).

While the PSA promoter maintains faithful tissue-specific expression, its promoter activity is relatively weak compared to the constitutive active RSV promoter (compare **Figure 5A** and **5E**). This implies that as a trade-off for the tissue specificity, the expression of a therapeutic transgene driven by the PSA promoter will be lower than that of a constitutively active promoter. This may not seem to be a major issue because we are using a conditional oncolytic strategy in which the therapeutic transgene itself is the Ad5 E1 gene. Theoretically, only low levels of E1 expression are required to initiate and maintain the viral oncolytic cycle to eradicate all the prostate cells. In this study, we have demonstrated that at an moi of 1, AdPSAE1 was able to completely eradicate all cancerous prostate cells *in vitro* (**Figure 2, 3** and **4**). Similarly, in our *in vivo* study, at viral doses (i.e., intratumoral injection of $5x10^6$ pfu AdPSAE1 per tumor of 200 mm^3 size, **Figure 6**) much lower than that of the typical E1-deleted adenoviral vectors we have routinely used (i.e., intratumoral injection of $5x10^9$ pfu E1-deleted adenovirus containing a therapeutic gene per tumor of 100 mm^3 size, Steiner et al, 2000b, 2000c), AdPSAE1 exhibited an equivalent inhibition ability for xenograft prostate tumor growth as those by E1-deleted adenovirus at a much higher dose. However, we were still unable to completely eradicate tumors using AdPSAE1 treatment *in vivo* (**Figure 6A**). This failure may be due to insufficient production of the E1 protein *in vivo* by the relatively weak prostate-specific promoter.

The limitation of this strategy by a PSA promoter driven, prostate-specific gene expression is that it only works effectively in PSA-producing prostate cells (such as LNCaP and PPC-1 as shown in this report), but not in

PSA-negative prostate cells such as DU145 and PC3 (Rodriguez et al, 1997; Yu et al, 1999b). Therefore, other prostate-specific promoters (such as probasin) should be explored for their abilities to drive transgene expression in PSA-negative prostate cancer cells. Our ongoing research showed that a 456-bp probasin promoter is able to drive transgene specifically expressed in both PSA-positive and PSA-negative prostate cancer cells. It implies that this 456-bp 5' region of the probasin gene might be a good candidate to function as a prostate-specific promoter to drive the E1 transgene expression in prostate cancer.

The idea of using conditional oncolytic viruses is an attractive strategy that may hold the promise of 100% eradication of primary tumor cells and of targeting tumor metastases. However, significant effort should be undertaken to evaluate the tissue specificity and ensure the safety of each new viral construct. A study to evaluate the biodistribution and toxicity of a replication-competent adenovirus following intraprostatic injection showed that although the virus persisted in the urogenital tract and liver, most toxicity was minimal and self-limiting. Most importantly, there was no germ-line transmission of viral genes (Paielli et al, 2000). One way to control viral spread is to design a conditional oncolytic virus containing a prodrug enzyme gene, so the prodrug can be used as desired to suppress viral replication effectively. A replication-competent, E1b-attenuated adenovirus containing a cytosine deaminase/herpes simplex virus type 1-thymidine kinase (CD/HSV-TK) fusion gene was constructed (Freytag et al, 1998). Not only the suicide gene system allows for the utilization of double-suicide gene therapy, but also it provides a means to eliminate the virus itself by destroying the host cells in situ and controls viral spread whenever needed (Freytag et al, 1998).

Recent development in this field has brought the hope closer to generate the ideal conditional replication-competent adenovirus for prostate cancer gene therapy. It appears that PSA prompter/enhancer has more activity and specificity in helper-dependent adenoviral vector (almost devoid of all adenoviral sequences) than in traditional E1-deleted adenoviral vector (Shi et al, 2002). Moreover, this promoter specificity can also be influenced by other constitutively active promoter/enhancer in the vector backbone (Shi et al, 2002). To overcome the obstacle that PSA promoter is active only in PSA-producing prostate cancer cells, a strategy of cotransduction of another adenovirus expressing androgen receptor (AR) and combination with dihydrotestosterone (DHT) treatment should be worth exploration. Because PSA promoter-driven transgene can be induced by DHT in PC-3 cells (a non-PSA-producing prostate cancer cell line) transfected with AR expression vector (Kizu et al, 2004). Moreover, a novel TARP (T cell receptor gamma-chain alternate reading frame protein) promoter with PSA enhancer has shown a high prostate-specific activity in both hormone-dependent and hormone-refractory prostate cancer cells (Cheng et al, 2004). With significant ongoing efforts of better understanding and improvement in these aspects, we expect that ideal conditional replication-competent adenoviruses will be generated and become an effective means for the treatment of prostate cancer in the near future.

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