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Recombinant Adenovirus Mediated Prostate-Specific Enzyme Pro-Drug Gene Therapy Regulated by Prostate-Specific Membrane Antigen (PSMA) Enhancer/Promoter

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ABSTRACT: Gene directed enzyme pro-drug therapy (GDEPT) is one of the adjuvant therapeutic regimens for advanced prostate adenocarcinoma, and this research intended to explore how to apply targeting therapy of prostate adenocarcinoma under the mediation of a promoter/enhancer of prostate-specific membrane antigen ($PSMA_{EP}$) as a specific regulatory element. Recombinant adenoviruses (Ad-$PSMA_{E\text{-}P}$–enhanced green fluorescent protein [EGFP], Ad-CMV-EGFP, Ad-$PSMA_{E\text{-}P}$–CD, and Ad-CMV-CD) were constructed and could express cytosine deaminase (CD) or the EGFP reporter gene driven by a $PSMA_{EP}$ or cytomegalovirus (CMV) promoter. LNCaP, CL-1, MCF-7, and A549 were infected with CD-produced recombinant adenoviruses and treated with pro-drug 5-fluorocytosine (5-FC) in vivo and vitro; then, the growth inhibition of the cells and the cell cycle variation were assessed by an [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay and flow cytometry. Growth suppression of the xenograft tumor was also adopted to evaluate the efficiency of the suicide system. Morphologic changes after treatment in vivo were assessed with hematoxylin and eosin staining. In the 4 examined cancer cell lines, PSMA-positive prostate cancer cells LNCap and CL-1 were exclusively sensitive to the Ad-$PSMA_{E\text{-}P}$–CD/5-FC system. The S phase of cell cycle arrest was thought to be involved in the cytotoxicity of 5-fluorouracil (5-FU) converted from 5-FC by CD. CL-1 implanted Athymic BALB/c mice showed growth inhibition of tumors when they were treated with the Ad-$PSMA_{E\text{-}P}$–CD/5-FC system without systemic conversion toxicity. The PSMA-based, CD-produced adenovirus, deserving further investigation in the future, might be a good candidate for targeting gene therapy of prostate adenocarcinoma.

Key words: Cytosine deaminase, prostate adenocarcinoma.

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Prostate cancer is one of the most common malignancies in men. Treatment for prostate cancer is based on androgen deprivation. Unfortunately, prostate cancer cells gradually show resistance to anti-androgen treatments in the progression of the disease. Radiation therapy might be a choice for hormone-refractory prostate cancer, but the efficacy of this treatment is associated with the dosage of radiation. To date, long-term complications from radiation occurred when the dosage was over 70 Gy (Freytag et al, 2003). It is necessary to find more efficient adjuvant therapies with less complication. Gene directed enzyme pro-drug therapy (GDEPT) is regarded as one of the potential therapeutic strategies. Undoubtedly, the increase of GDEPT's efficiency depends on the achievement of the following 3 significant aspects: 1) target gene expression, specifically in tumor cells; 2) high transduction efficiency of vectors; and 3) strong toxicity of drugs converted from nontoxic drugs. In the past decade, many researches have been involved in the improvement of GDEPT's efficiency in various tumors (Chung-Faye et al, 2001; Miller et al, 2002; Li et al, 2003; Miyagi et al, 2003).

For broad suicide gene therapy, most work has been focused on cytosine deaminase/5-fluorocytosine (CD/5-FC) and herpes simplex virus thymidine kinase/ganciclduiv (HSV-tk/GCV) systems. The CD gene, an enzyme, present in fungi and bacteria but absent from mammalian cells, could convert cytosine into uracil. CD could also deaminate the nontoxic pro-drug 5-FC to its highly toxic derivative 5-FU, which is then converted by cellular enzymes into 5-FUTP and 5-Fluoro-dUMP (5-FdUMP). 5-FUTP could be incorporated into RNA to replace UTP, resulting in the inhibition of the nuclear processing of recombinant and messenger RNAs (rRNAs and mRNAs), whereas 5-FdUMP could

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irreversibly inhibit thymidylate synthetase to prevent DNA synthesis. The CD/5-FC system has been widely applied in studies of various tumors in the past years because of its predominantly bystander effect compared with other suicide gene systems (Ichikawa et al, 2000). Advanced approaches have utilized regulatory elements of several specific tumor markers, including prostate-specific antigen (PSA), HSV-tk/GCV carcinoembryonic antigen (CEA), and α-fetoprotein (AFP), to achieve target gene expression in tumor cells exclusively (Tanaka et al, 1996; Spitzweg et al, 1999; Huang et al, 2002).

Several prostatic-specific markers, such as PSA, human glandular kallikrein 2 (hk2), probasin, and prostate androgen-regulatory gene (PAR), have been applied to targeting gene therapy. Results of these experiments showed that these markers could mediate prostate-specific therapeutic effects (Spitzweg et al, 1999; Yu et al, 1999; Xie et al, 2001; Xu et al, 2006), but the prostate-specific regulatory elements mentioned above were down-regulated by androgen deprivation (Israeli et al, 1994), which limited the effect on advanced or metastatic prostate adenocarcinoma. Prostate-specific membrane antigen (PSMA), a new prostate-specific marker, is a type II transmembrane glycoprotein. It has been confirmed to have high expression in advanced or metastatic prostate adenocarcinoma and is positively related to the Gleason score (Wright et al, 1996). PSMA may be a better element for targeting gene therapy in advanced and metastatic prostate adenocarcinomas. O'Keefe et al (2000) reported that they have verified the specific transcriptional activity of the PSMA enhancer/promoter transduced by the plasmid vector, but the efficiency of the PSMA enhancer/promoter by use of adenoviral technology is still unknown. In contrast to nonviral vectors, adenovirus possesses a higher transduction efficiency, a larger capacity for exogenous genes, and a more stable exogenous gene expression (Russell, 2000).

We previously have constructed and analyzed the transcriptional regulatory elements of PSMA and have chosen the best functional element combination of PSMA promoter and enhancer successfully (Zeng et al, 2005). In the present study, we just planned to construct a CD gene that expressed recombinant adenovirus regulated by the PSMA promoter/enhancer and expected to observe the specific cytotoxicity of the Ad-$PSMA_{E\text{-}P}$–CD/5-FC system in prostate adenocarcinomas.

Materials and Methods

Cell Lines and Culture

Human androgen-dependent prostate cancer cell line LNCap, human androgen-independent prostate cancer cell line CL-1, human breast cancer cell line MCF-7, and human lung adenocarcinoma cell line A549 were purchased from ATCC (Rockville, Md). HEK293 was gained from AdEasy vector system (Qbiogene, Carlsbad, Calif). LNCap, CL-1, and MCF-7 cell lines were maintained at 37°C in a complete medium of RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO, Rockville, Md), while A549 and HEK293 cells were maintained in Dulbecco modified Eagle medium with 5% fetal bovine serum (GIBCO). One hundred units per milliliter of penicillin and 100 ng/ml of streptomycin were added to all media unless otherwise stated.

Reagents and Plasmids

Plasmid p enhanced green fluorescent protein (EGFP)-$PSMA_{E\text{-}P}$ was constructed previously (Zeng et al, 2005). The recombinant adenovirus vector system was purchased from Qbiogene. The LA-*Taq* DNA polymerase kit and reverse transcriptase–polymerase chain reaction (RT-PCR) reagents were obtained from TakaRa Biotech (Tokyo, Japan). Restricted endonucleases and other modified nucleases were from TOYOBO (Osaka, Japan). Transfection reagent was Lipofectamine2000 (Invitrogen, Carlsbad, Calif). PCR purification and gel extraction kits were from Omega Bio-tek (Doraville, Ga). The 5-FC and 5-FU were from Sigma Chemical Co (St Louis, Mo).

Recombinant Adenovirus Vectors

Recombinant replication-defective Ad vectors were prepared, purified, and titrated according to the manual of the AdEasy vector system (Qbiogene) (Figure 1). Briefly, the recombinant adenoviruses were propagated in HEK293 cells and purified by the continuous CsCl centrifugation method. The purified virus stock was then dialyzed against 10 mM Tris buffer, pH 7.5, containing 1 mM $MgCl_2$ and 10% glycerol, and the concentrated virus was titrated, aliquoted, and stored at −80°C. Four recombinant replication-defective Ad vectors in the experiment were Ad-$PSMA_{E\text{-}P}$–CD, Ad-CMV-CD, Ad-$PSMA_{E\text{-}P}$-EGFP, and Ad-CMV-EGFP, with the therapeutic toxic gene *E coli* CD (GenBank accession number S56903) or a reporter EGFP driven by a CMV promoter or PSMA promoter (Zeng et al, 2005). Virus titers were determined by the tissue culture infectious dose 50 ($TCID_{50}$) method and OD_{260} method according to the manual of the AdEasy vector system (Qbiogene).

Quantification of Adenovirus-Specific Efficiency

Adenovirus efficiency was quantified by monitoring the expression of EGFP in Ad-CMV-GFP or Ad-$PSMA_{E\text{-}P}$-EGFP infected cells. Briefly, cells (0.5×10^6 to 1×10^6) were plated in 6-well plates, allowed to adhere overnight, and subsequently infected with Ad-CMV-GFP or Ad-$PSMA_{E\text{-}P}$-EGFP at various multiplicities of infection (MOIs) (0–200 PFU/cell). Twenty-four hours after infection, EGFP expression was assayed with a fluorescence microscope (Olympus, Tokyo, Japan), and then the cells were harvested with 0.25% trypsin/EDTA, washed with buffer (phosphate-buffered saline, 0.1% sodium azide, and 0.1% bovine serum albumin), and analyzed by flow cytometry (Coulter Elite Esp), which was repeated 3 times.

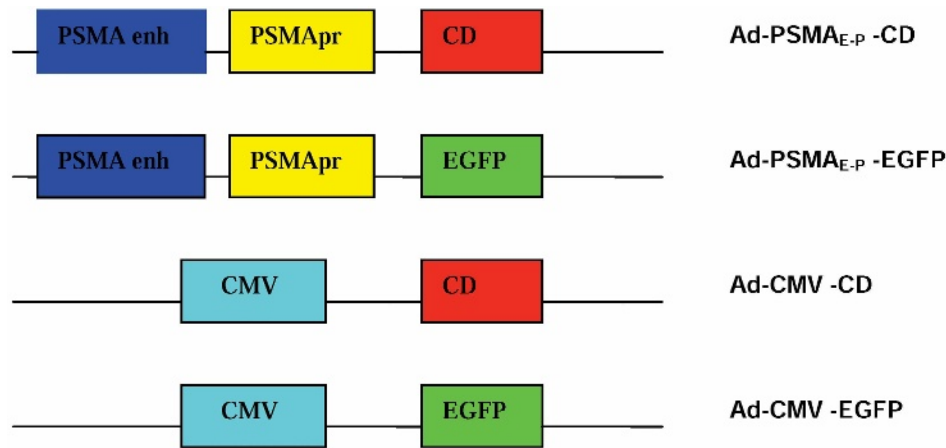

Figure 1. Diagram of recombinant adenoviruses construction. Blue square indicates PSMA enhancer; yellow square, PSMA promoter; light blue square, CMV promoter; red square, CD gene; and green square, EGFP gene.

Ad-PSMA$_{E-P}$–CD/5-FC and Ad-CMV-CD/5-FC Toxicity

Four types of cells, including LNCap, CL-1, A549, and MCF-7, were harvested from confluent monolayers and plated at 1.5 × 10^6 to 3 × 10^6 cells/well in T25 flasks overnight at 37°C. Cells were infected with various amounts of Ad-CMV-CD or Ad-PSMA$_{E-P}$–CD (0–200 PFU/cell). Twenty-four hours after infection, cells were harvested with 0.25% trypsin/EDTA, plated into 96-well tissue culture plates at 5000 cells/well in 100 μl of complete media, and allowed to adhere overnight at 37°C. One hundred microliters of media supplemented with serial dilutions of 5-FC (6 replicates/dilution) was added, and cells were further incubated for 2–8 days. Cell survival was determined by an MTT assay. Fractional cell survival data were plotted against the logarithm of drug concentration, and 50% infective concentration (IC_{50}) values were extrapolated by piecewise linear regression; cell survival status following different end points was also determined.

Bystander Effect of Viral System

CL-1 cells were exposed to Ad-CMV-CD or Ad-PSMA$_{E-P}$–CD under conditions that could lead to 100% infection of cells. The infected cells were then mixed with noninfected cells at a different ratio and subjected to 5-FC at a concentration of 15 μmol/L. The percentages of infected cells in the mixtures were 0%, 10%, 20%, 30%, 40%, and 100%. Mixed cells were plated in 6-well plates, and media were changed every 3 days. After 10 days' culture, cells were counted by staining with typan blue.

In Vivo Toxicity of Ad-CD/5-FC System

CL-1 and A549 tumors were established by injecting 1 × 10^7 cells into 5-week-old athymic BALB/c male mice (Animal Center, Sichuan University, People's Republic of China), and all cells were inoculated subcutaneously in the right flank of the mice. Two weeks later, the size of the tumors reached 8–12 mm in diameter. Xenograft mice were randomly divided into 6 groups for CL-1 injected mice and 2 groups for A549 injected mice (5 mice/group) and then treated with recombinant adenoviruses (Ad-CMV-CD or Ad-PSMA$_{E-P}$–CD) every 6 days (intratumoral injection, 2 × 10^9 PFU/0.2 ml). From the second day after adenovirus injection, mice were further treated with 5-FC (500 mg/kg) or saline every other day (intraperitoneal injection, every 2 days). Sizes of tumors in each mouse were measured every 4 days, and volumes were calculated with the following formula: volume = length × width^2 × 0.52 (mm^3). Four weeks later, mice were sacrificed. Tissues, including xenograft tumor, liver, and kidney, were fixed and then stained with hematoxylin and eosin.

Statistical Analysis

Statistical analysis was performed by SPSS 13.0 software (SPSS Inc, Chicago, Ill). $P < .05$ was considered statistically significant.

Results

Transcriptional Activity of Constructed Recombinant Adenoviruses

Replication-defective adenovirus Ad-PSMA$_{E-P}$–EGFP and control adenovirus Ad-CMV–EGFP were compared in prostate cancer cell line LNCap and CL-1, breast cancer cell line MCF-7, and lung cancer cell line A549. The EGFP expression in different cell lines was examined by fluorescence and flow cytometry (Figure 2). Ad-CMV-EGFP virus expressed a high level of EGFP in all of 4 types of cell lines. In contrast, Ad-PSMA$_{E-P}$–EGFP could express only a detectable level of EGFP in LNCap and CL-1 cell lines. EGFP expression in LNCap and CL-1 cells infected with Ad-PSMA$_{E-P}$–EGFP was weaker than that in cells infected with Ad-CMV-EGFP. Flow cytometry showed that the PSMA-based adenovirus possessed only about 20% of the transcriptional activity of the CMV-based control (Figure 3).

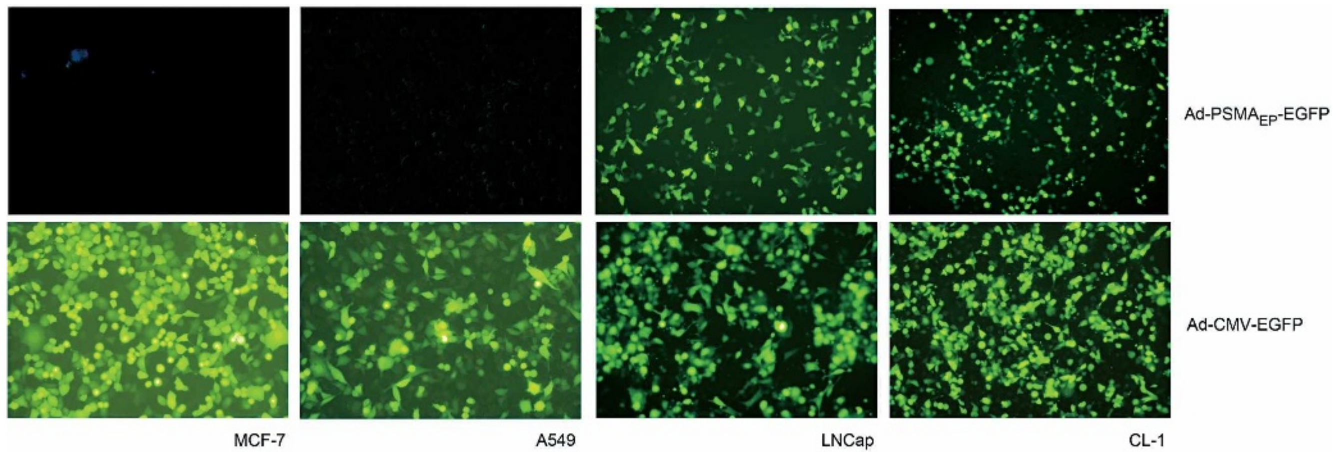

Figure 2. Expression of EGFP in MCF-7, A549, CL-1, and LNCap cell lines following infection of cells with either Ad-CMV-EGFP or Ad-PSMA$_{E\text{-}P}$–EGFP. Cells were plated at 10^5 cells/well in a 96-well plate and infected with recombinant adenoviruses at a MOI of 100 PFU/cell. After 48 hours, EGFP expression was observed under fluorescence microscopy (magnification 100×).

Specific Toxicity of Ad-PSMA$_{E\text{-}P}$–CD/5-FC System to Prostate Cancer Cell Line

The 5-FC sensitivity (IC_{50} in millimoles per liter) varied according to tumor cell types: 0.238, 0.236, 1.75, and 2.73 mmol/L in LNCap, CL-1, MCF-7, and A549, respectively. Ad-PSMA$_{E\text{-}P}$–CD/5-FC and Ad-CMV-CD/5-FC toxicity (5-FC IC_{50} in millimoles per liter) were assessed with 4 cell lines after infection with Ad-PSMA$_{E\text{-}P}$–CD or Ad-CMV-CD at 0, 10, 50, 100, and 200 MOI (PFU/cell). Dose-response curves from LNCap, CL-1, MCF-7, and A549 were shown in Figure 4, and a significant linear relation ($P < .01$) between Ad-CMV-CD MOI and 5-FC sensitivity (log-log scale) for each of the 4 cell lines was observed. Similar results were obtained between Ad-PSMA$_{E\text{-}P}$–CD MOI and 5-FC sensitivity but only for the LNCap and CL-1 cell lines. The 5-FC sensitivity of the LNCap and CL-1 cell lines (infected with Ad-CMV-CD at 100 MOI) increased 314- to 518-fold higher than that of uninfected cells. Although Ad-PSMA$_{E\text{-}P}$–CD/5-FC could only increase the 5-FC sensitivity of LNCap and CL-1 by 70- to 134-fold; cytotoxicity induced by Ad-PSMA$_{E\text{-}P}$–CD was exclusively detected in PSMA-positive prostate cancer cell lines, which indicated the high specificity of the suicide gene system.

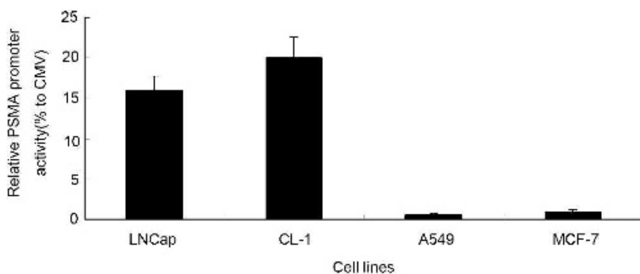

Figure 3. Comparison of EGFP expression level following infection of MCF-7, A549, CL-1, and LNCap cell lines with Ad-PSMA$_{E\text{-}P}$–EGFP; parallel control infection was carried out with Ad-CMV-EGFP.

In Vitro Effect of Ad-PSMA$_{E\text{-}P}$–CD/5-FC System

Cytotoxicity was examined in vitro by infecting 4 types of cells with either Ad-PSMA$_{E\text{-}P}$–CD or Ad-CMV-CD at a MOI of 100 added in 15 μmol/L 5-FC (the concentration was selected because higher multiplicities of infection resulted in the nonspecific killing of cells due to viral infection [data not shown]). Figure 5 shows that the sensitivity of 4 cells to the pro-drug 5-FC can obviously be enhanced by infecting them with Ad-CMV-CD in vitro. However, Ad-PSMA$_{E\text{-}P}$–CD/5-FC could only enhance sensitivity to 5-FC in PSMA-positive prostate cancer cell lines (LNCap and CL-1). We assumed that this CD suicide gene therapeutic system was uniquely effective in prostate cancer cells.

Flow Cytometry Assay

Cell cycle variations were determined by flow cytometry. Results indicated that both Ad-CMV–CD/5-FC and Ad-PSMA$_{E\text{-}P}$–CD/5-FC systems could arrest cell accumulation before S phase to inhibit cell growth in LNCap and CL-1 cells (Figure 6a showed cell cycle variation in LNCap). In contrast, in MCF-7 and A549 cells, only the Ad-CMV–CD/5-FC system could arrest cells entering the S phase (Figure 6b showed cell cycle variation in A549).

Bystander Effect

A total of 30% of the Ad-PSMA$_{E\text{-}P}$–CD infected CL-1 cells treated with 15 μmol/L 5-FC for 8 days were shown to kill 100% of the parental tumor cells (Figure 7), while 10% of the Ad-CMV–CD infected CL-1 cells could lead

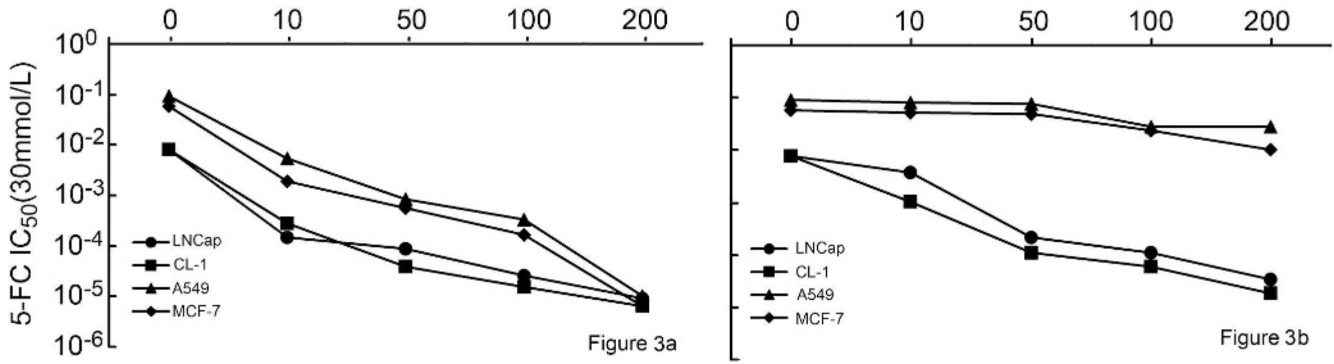

Figure 4. Ad-CMV-CD/5-FC **(a)** and Ad-PSMA$_{E-P}$–CD /5-FC **(b)** dose response. The sensitivity of 5-FC (IC_{50} in millimoles per liter) was quantified with cells infected with increasing MOIs (PFU/cell) of Ad-CMV-CD or Ad-PSMA$_{E-P}$–CD /5-FC, and the dose response was determined. All the data were represented as the mean value of 3 duplications.

to 100% cell death. These data showed that cells could be sensitized, even though less than 100% of the cells were infected.

In Vivo Cytotoxicity

Ad-CMV–CD/5-FC and Ad-PSMA$_{E-P}$–CD/5-FC injected mice, which were implanted with CL-1, showed different extents of growth inhibition ratio (61.5% vs 49.4%), while in A549 implantation groups, the xenograft was sensitive only to the Ad-CMV–CD/5-FC system (shown in Figure 8). On day 21 after treatment, compared to the control groups, the sizes of the tumor in the Ad-CMV–CD/5-FC and Ad-PSMA$_{E-P}$–CD/5-FC treated groups were only 1952 ± 427 mm^3 and 2568 ± 487 mm^3, respectively. At the same time, in A549 implanted mice, the volume of tumor could be suppressed only in the Ad-CMV–CD/5-FC treated group (Table). In paraffin-embedded tissue sections from sacrificed mice with hematoxylin and eosin staining indicated, the Ad-CMV–CD/5-FC system showed systemic toxicity. Necrosis was predominantly observed in the liver tissue, while necrosis in the kidney was relatively mild. On the other hand, the toxicity of the Ad-PSMA$_{E-P}$–CD/5-FC system was confined to the PSMA-positive tumor cells (Figure 9).

Discussion

For patients with prostate adenocarcinoma, a radical prostatectomy alone or in combination with irradiation and endocrine treatment is the standard and curative regimen. Unfortunately, most prostate adenocarcinomas in China have extended out of capsule before treatment, so that tumors would ultimately develop into hormone-refractory disease. The current therapeutic

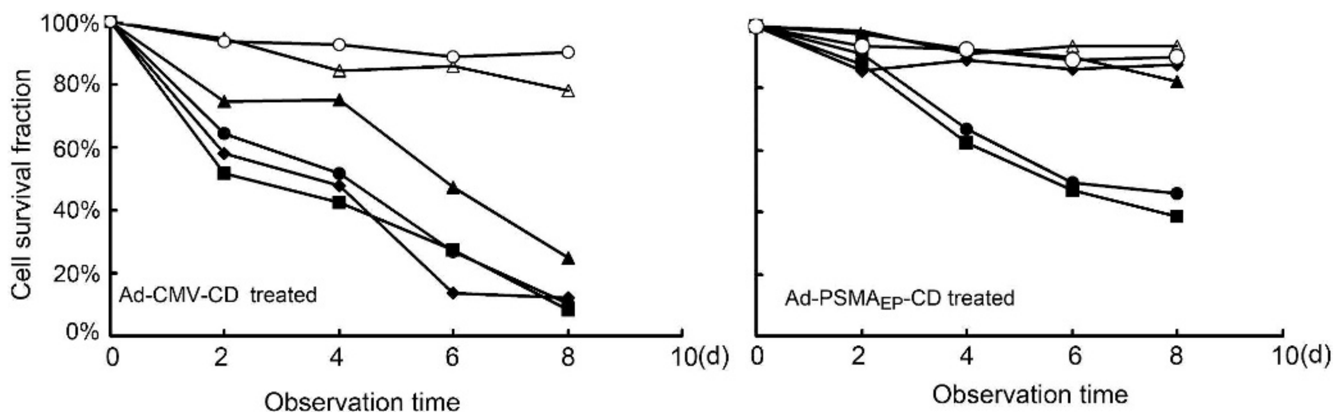

Figure 5. Cytotoxicity of LNCap, CL-1, A549, and MCF-7 treated with the Ad-PSMA$_{E-P}$-CD/5-FC or Ad-CMV-CD system. All of the cells were plated in 96-well plates, infected with Ad-PSMA$_{E-P}$-CD or Ad-CMV-CD (MOI: 100 PFU/cell). The next day, cells were replaced with fresh complete media with added 5-FC (15 μmol/L) and further incubated for 2, 4, 6, and 8 days separately; cell survival ratios were assessed by the MTT assay. All the data were represented as the mean value of 3 assays. ● represents LNCap treated with Ad-PSMA$_{E-P}$-CD or Ad-CMV-CD plus 5-FC; ■ represents CL-1 treated with Ad-PSMA$_{E-P}$-CD or Ad-CMV-CD plus 5-FC; ▲ represents A549 treated with Ad-PSMA$_{E-P}$-CD or Ad-CMV-CD plus 5-FC; ◆ represents MCF-7 treated with Ad-PSMA$_{E-P}$-CD or Ad-CMV-CD plus 5-FC; Δ represents CL-1 treated with Ad-PSMA$_{E-P}$-CD or Ad-CMV-CD alone; ○ represents CL-1 treated with 5-FC alone.

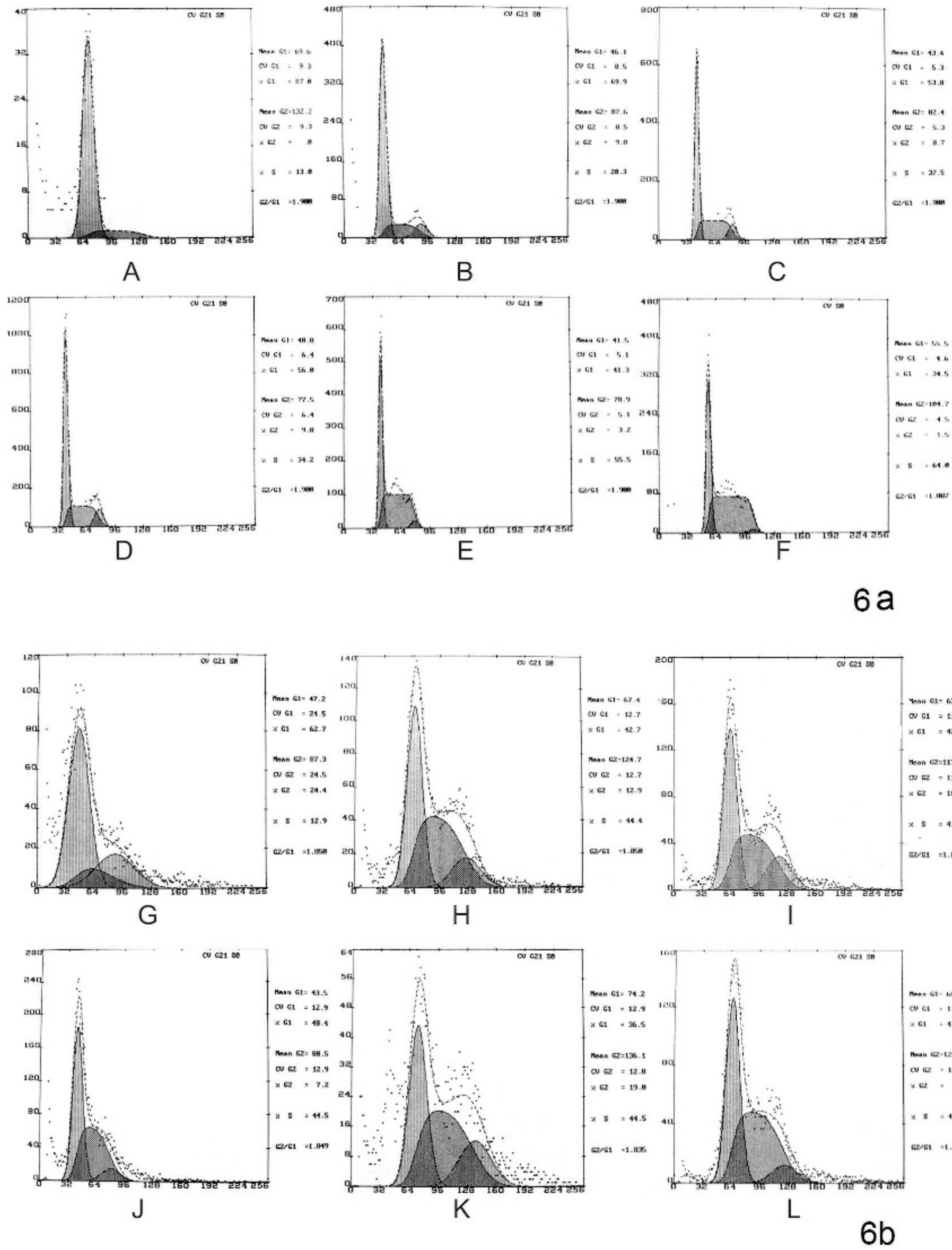

Figure 6a. Cell cycle variations of LNCap with different managements. **(A)** LNCap dealt with Ad-CMV-CD (MOI: 100) plus 5-FC (concentration: 15 μmol/L), ratio of S phase 13%; **(B)** LNCap dealt with Ad-PSMA$_{E-P}$-CD (MOI: 100) plus 5-FC (concentration: 15 μmol/L), ratio of S phase 20.3%; **(C)** LNCap infected with Ad-PSMA$_{E-P}$-CD (MOI: 100) only, ratio of S phase 37.5%; **(D)** LNCap infected with Ad-CMV-CD (MOI: 100) only, ratio of S phase 34.2%; **(E)** LNCap treated with 5-FC (concentration: 15 μmol/L) only, ratio of S phase 55.5%; **(F)** LNCap without any management as control, ratio of S phase 64%.Figure 6b. Cell cycle variations of A549 with different managements. **(G)** A549 dealt with Ad-CMV-CD (MOI: 100) plus 5-FC (concentration: 15 μmol/L), ratio of S phase 12.9%; **(H)** A549 dealt with Ad-PSMA$_{E-P}$-CD (MOI: 100) plus 5-FC (concentration: 15 μmol/L), ratio of S phase 44.4%; **(I)** A549 infected with Ad-CMV-CD (MOI: 100) only, ratio of S phase 41.5%; **(J)** A549 infected with Ad-PSMA$_{E-P}$-CD (MOI: 100) only, ratio of S phase 44.5%; **(K)** A549 treated with 5-FC (concentration: 15 μmol/L) only, ratio of S phase 44.5%; **(L)** A549 without any management as control, ratio of S phase 48.7%.

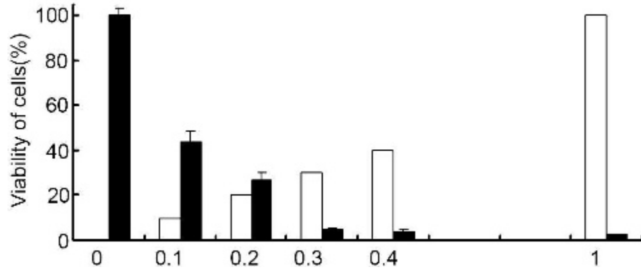

Figure 7. Bystander effect of Ad-PSMA$_{E\text{-}P}$-CD/5-FC system. Blank column indicates viability of CL-1 cells; white column, ratio of Ad-PSMA$_{E\text{-}P}$-CD infected CL-1 cells.

approaches could only be palliative. Gene therapy and immunotherapy may be novel adjuvant treatments for advanced and metastatic prostate adenocarcinomas.

As one of the ideal models for gene therapeutic research, several prostate-specific gene promoter elements have been studied, such as PSA, hk2, and probasin. However, it deserves attention that PSA, hk2, or probasin is down-regulated by androgen deprivation (Israeli et al, 1994). Hormone-refractory prostate adenocarcinomas might become insensitized to gene therapeutic systems based on PSA, hk2, and probasin. Prostate-specific membrane antigen is a novel prostate-specific marker in prostate adenocarcinoma, which was first identified in the frozen tissue of prostate cancer early in 1987. The PSMA expression level in the prostate is 1000-fold higher than that in nonprostate tissues. Further studies have demonstrated that PSMA expression in advanced or metastatic prostate adenocarcinomas is several times higher than in nonmalignant prostate tissues (Israeli et al, 1994; Silver et al, 1997). It is exciting that PSMA expression is up-regulated by androgen deprivation (Wright et al, 1996), which indicates that PSMA is an excellent target gene in the therapy of hormone-refractory prostate adenocarcinomas. Early in 2000, O'Keefe et al utilized PSMA promoter and enhancer to regulate CD gene expression mediated by plasmids, and their result showed the specific expression and conversion potential of CD controlled by PSMA regulatory elements (O'Keefe et al, 2000; Uchida et al, 2001). Lee et al (2002) constructed a novel prostate-specific promoter PSES derived from the PSA and PSMA promoter/enhancer, which produced high selective activity in prostate cancer cells. The infectivity of tumor cells transfected with nonviral vectors is lower than that of viral vectors; lower transduction efficacy leads to a weaker therapeutic effect. The effect of CD-produced adenovirus mediated by PSMA regulatory elements is still unclear and deserves examination.

In the past 2 years, our group has screened and chosen the best regulatory element combination of PSMA enhancer and promoter (Zeng et al, 2005). We first constructed CD-produced and reporter gene (EGFP)-produced recombinant adenoviruses controlled by the prostate-specific regulatory element chosen previously. EGFP expression in vitro appeared exclusively in PSMA-positive prostate cancer cell lines when cells were infected with Ad-PSMA$_{E\text{-}P}$–EGFP, demonstrating the specific regulatory function of the PSMA enhancer/promoter repeatedly. Only LNCap and CL-1 cells were sensitized to the Ad-PSMA$_{E\text{-}P}$–CD/5-FC system in the 4 examined cells, while the Ad-CMV–CD/5-FC system showed its versatility to all cell lines. The sensitivity of 5-FC to LNCap and CL-1 cells infected with Ad-PSMA$_{E\text{-}P}$–CD predominantly increased 70- to 134-fold higher than that of uninfected cells. The CL-1 cell line, derived from LNCap, was one of the androgen-independent prostate cancer cell lines, which also expressed PSMA. In the present study, the results demonstrated that the Ad-PSMA$_{E\text{-}P}$–CD/5-FC system could lead to cytotoxicity in PSMA-positive cells (LNCap and CL-1), whether it was androgen-dependent or not. Although we did not compare the effect of Ad-PSMA$_{E\text{-}P}$–CD with the PSMA$_{E\text{-}P}$-based CD gene-expressed plasmid parallel in our experiment, compared with data from O'Keefe et al (2000), the transduction efficiency of adenovirus was definitively better than that of plasmids. According to statistic analysis, we found that the 4 tested cells maintained different sensitivities to the Ad-

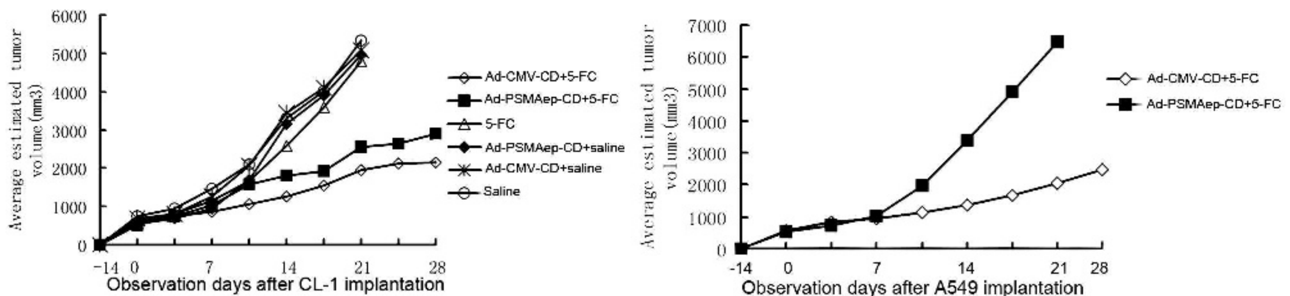

Figure 8. Growth suppression of CL-1 and A549 in vivo injected with 2 types of adenoviral vectors. All the data were represented as the mean value of 5 individual determinations.

Tumor size of each groups before and after treatment

Group*	Volume of tumor, mm^3 (mean ± SD)	
	Day 0	Day 21
CL-1 implantation	...	...
Ad-CMV-CD + 5-FC	637 ± 91	1952 ± 427
Ad-PSMA$_{E-P}$–CD + 5-FC	516 ± 105	2568 ± 487
Ad-PSMA$_{E-P}$–CD + saline	533 ± 94	4975 ± 713
Ad-CMV-CD + saline	616 ± 110	5072 ± 658
5-FC only	572 ± 102	4784 ± 634
Saline only	584 ± 96	5458 ± 726
A549 implantation	...	...
Ad-CMV-CD + 5-FC	567 ± 113	2045 ± 443
Ad-PSMA$_{E-P}$–CD + 5-FC	533 ± 98	6455 ± 735

* In CL-1 implanted groups, after treatment, there was no difference between Ad-CMV-CD+5-FC and Ad-PSMA$_{E-P}$–CD+5-FC groups (P = .305); however, there were significant differences among Ad-CMV-CD+5-FC, Ad-PSMA$_{E-P}$–CD+5-FC, and control groups ($P < .0001$). In A549 implanted groups, there was significant difference between Ad-CMV-CD+5-FC and Ad-PSMA$_{E-P}$–CD+5-FC groups (P = .0003).

CMV–CD/5-FC system, which may be because of different densities of coxsackievirus and adenovirus receptor upon the extracellular surface (Kraaij et al, 2005). Compared with the CMV promoter, the regulatory activity of the PSMA enhancer/promoter was indeed lower, whereas Ad-PSMA$_{E-P}$–CD/5-FC could actually achieve targeting cytotoxicity to PSMA-positive cancer cells.

In addition, the flow cytometry assay confirmed that cell arrest before the S phase was involved in the growth inhibition of LNCap or CL-1 cells treated with Ad-PSMA$_{E-P}$–CD or Ad-CMV–CD plus 5-FC. The ratio of cell arrest before S phase in LNCap cells infected with Ad-PSMA$_{E-P}$–CD was lower than that of LNCap cells infected with Ad-CMV–CD (69.9% vs 87%); however, it did not appear to affect the cytotoxicity of the Ad-PSMA$_{E-P}$–CD/5-FC system in PSMA-positive cells. Although the adenoviral vector has a higher infectivity than the plasmid vector, it was impossible to infect 100% of the tumor cells. In the present study, the bystander effect of adenovirus was also observed; the results verified the significant effect of this kind of phenomenon beyond doubt. The present study confirmed the specific cytotoxic effects of the Ad-PSMA$_{E-P}$–CD/5-FC system in vivo, and the tumor growth of mice implanted with CL-1 cells decreased almost 50% during the observation time. Compared with the systemic

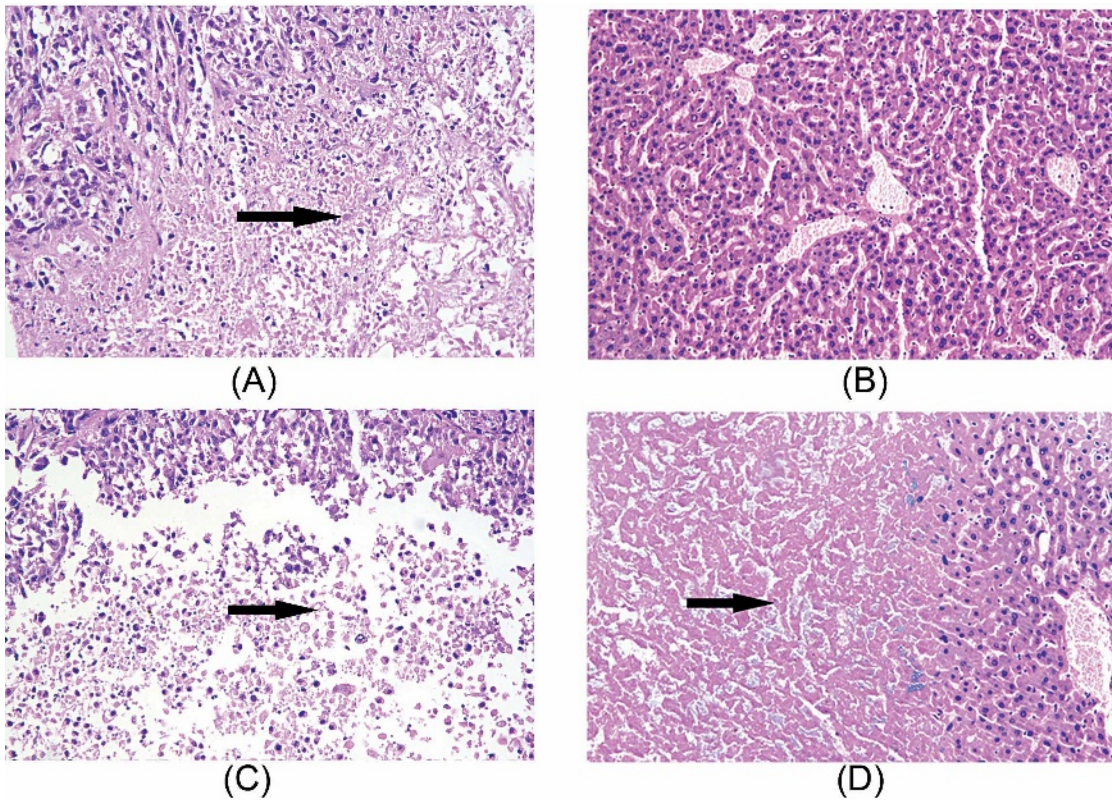

Figure 9. Tissue responses to the Ad-PSMA$_{E-P}$-CD/5-FC and Ad-CMV-CD/5-FC systems. **(A)** The CL-1 tumor dealt with the Ad-PSMA$_{E-P}$-CD/5-FC system. Hematoxylin and eosin staining showed obvious necrosis in the tumor cells. **(B)** Liver tissue dealt with the Ad-PSMA$_{E-P}$-CD/5-FC system. Hematoxylin and eosin staining showed no cytotoxic pathological changes. **(C)** The CL-1 tumor dealt with the Ad-CMV-CD/5-FC system. Hematoxylin and eosin staining also showed necrosis in the tumor cells. **(D)** Liver tissue dealt with Ad-CMV-CD/5-FC system. Hematoxylin and eosin staining showed cytotoxic pathological changes (magnification 200×).

toxicity of the Ad-CMV–CD/5-FC system, the Ad-$PSMA_{E\text{-}P}$–CD/5-FC system exhibited its toxicity only in PSMA-positive tumor cells without adverse results to other organs, such as the liver and kidney. In brief, the Ad-$PSMA_{E\text{-}P}$–CD/5-FC system could effectively delay tumor growth in vivo without systemic toxicity.

Our experimental research confirmed the specific transcriptional activity of the PSMA enhancer/promoter in vivo and in vitro, and we first constructed Ad-$PSMA_{E\text{-}P}$–CD/5-FC system. The transduction efficiency of the recombinant adenovirus might be better than the general plasmid vector in targeting gene therapy. PSMA is worth being investigated in depth in gene therapy for prostate adenocarcinoma.

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