Antitumor effect of dendritic cells transfected with prostate-specific membrane antigen recombinant adenovirus on prostate cancer: An in vitro study

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Abstract. The aim of the present study was to construct a dendritic cell (DC) vaccine transfected with a prostate-specific membrane antigen (PSMA) recombinant adenovirus and to observe the ability of the recombinant DCs in eliciting a PSMA-directed T-cell response to prostate cancer (PCa) in vitro. Replication-defective adenoviral vectors, were constructed using the Adxsi system. The virus titer was measured by TCID50 assay, and the expression of the PSMA gene was identified by polymerase chain reaction (PCR) generation of peripheral blood mononuclear cell (PBMC) derived DCs in vitro. In addition, a PE-7AAD apoptosis and necrosis kit was applied to detect the survival of DCs at different MOI values to determine the optimal MOI. Morphological changes of DC were observed under a fluorescence microscope, expression of the PSMA protein was detected by western blotting 48 h after transfection, the expression of DC markers prior to and following transfection was detected by direct immunofluorescence, and the interleukin (IL)-12 concentration in the culture supernatant of the three groups was measured by ELISA. The antitumor effect of DC-stimulated cytotoxic T lymphocytes (CTLs) on different PCa cell lines was analyzed using a Cell Counting Kit-8 assay. The optimal MOI value was determined to be 200. The PSMA protein was expressed in DCs, and the recombinant adenovirus did not impact the maturation and morphological changes of the DCs. The expression levels of the co-stimulatory molecules, CD80, CD83, CD86 and HLA-DR, were significantly higher in the Ad-PSMA-DC group than in the other two groups (P<0.05). The concentration of IL-12 in the supernatant of the Ad-PSMA-DC group (79.51±1.60 pg/ml) was significantly higher than the non-transfection group (P<0.05). The optimal effector to target (E:T) ratio was determined to be 40:1. However, at the same E:T ratio, the cytotoxic activity of PSMA-DC-T against the LNCap cells was markedly stronger than its activity against the other target cells (DU145 and 22RV; P<0.05); furthermore, the cytotoxic activity of PSMA-DC-T against the LNCap cells was significantly higher than the anti-LNCap effect of DC-T cells in other groups (P<0.05). In vitro experiments indicated that mature DCs transfected with Ad-PSMA secreted high concentrations of IL-12 and elicited potent antitumor immune responses targeting PSMA-expressing cells by stimulating the cytotoxic activity of CTLs.

Introduction

In the history of tumor immunotherapy, dendritic cell (DC) vaccines targeting cancer cells have been a significant focus of investigation (1). Abundant animal experiments, and phase I and II clinical trials have demonstrated that DCs elicit specific antitumor immune responses, which lead to tumor regression or stabilization (2). DC vaccines have entered phase I or II in clinical trials regarding the treatment of various types of tumor (1).

Prostate cancer (PCa) is the second most common type of malignancy in men in western countries (3) and the incidence of PCa in China has recently risen (4). Treatment of PCa predominantly comprises of surgery, radiotherapy, chemotherapy and endocrine therapy, which exert certain therapeutic effects, yet are accompanied by serious adverse reactions, particularly to patients exhibiting sex hormone-independent PCa, and recurrence and/or metastasis.

DC vaccine therapy has become a significant focus for PCa immunotherapy and also in the field of biotherapy as a whole. Studies in China and worldwide have evaluated DC vaccines [loaded with prostate-associated antigen peptides, including prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), prostatic acid phosphatase (PAP), prostate stem cell antigen, related recombinant protein and tumor cell lysates] in preclinical studies, and confirmed their safety and therapeutic effect of stimulating specific immune responses against PCa (5-7). Despite the progression of the DC vaccine in basic and clinical research, various issues remain to be clarified, such as the instability of the therapeutic effect, the selection of an appropriate tumor antigen, the route and dose

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of reinfused DCs, the selection of a gene transfection vector, and the extension of the response time of the vaccine-induced cytotoxic T lymphocyte (CTL). The present study hypothesizes that the DC vaccine presents a novel therapeutic method for the treatment of PCa, particularly for individuals with hormone resistance.

Currently, there are two strategies for constructing DC vaccines (8): i) DCs loaded with tumor antigen; and ii) DCs modified with cytokines. The former aims to elevate the targeting effect of immunotherapy, and predominantly consists of DCs pulsed with tumor antigens and DCs pulsed with antigen coding genes. However, the latter focuses on improving the efficacy of DC vaccines by introducing co-stimulatory molecules, cytokines and chemokines into the DCs. Of these methods, DCs modified by antigen coding genes effectively present tumor antigens to T cells subsequent to stimulation and continue to release endogenous tumor antigens. Therefore, DCs modified by antigen coding genes are considered to be more effective in stimulating T lymphocytes (in terms of a longer duration of antigen presenting and an improved targeting effect) when compared with DCs pulsed directly by tumor antigens or tumor cell debris. In addition, numerous studies have identified the association between DC function and a variety of cytokines, co-stimulatory molecules, and chemokines (9-11). Therefore, introducing various cytokines into DCs in order that they continue to express these factors has become an additional method of promoting DC maturation, improving DC activity, enhancing the antigen-presenting function (by increasing the expression level of co-stimulatory molecules), and strengthening the chemotaxis of DCs to T cells.

PSMA is a type of PCa marker, which is more sensitive and more specific when compared with PSA and PAP. Its highly specific expression in PCa tissues has rendered it a primary target for targeted therapy and a specific focus within PCa research (12). DCs are considered to be the most potent antigen-presenting cells in current knowledge. DCs stimulate and sensitize naïve T cells in the initiation of an early immune response. Furthermore, DCs elicit immunization targeting PCa cells upon ingestion and processing of tumor antigens, and presentation to T cells (13).

The selection of a vector is another key issue in the construction of DC vaccines targeted to PCa cells. Currently, gene therapy vectors consist of two major categories, viral vectors (adeno-viruses, retroviruses, aden-associated viruses, lentiviruses and the herpes simplex virus) and non-viral vectors (bacterial vectors, artificial carriers and liposome vectors). Non-viral vectors have no limitations in the size of inserted elements, possess low immunogenicity and are easy to produce; however, their low transfection efficiency in vivo has reduced their popularity compared with viral vectors (14). Among the widely used viral vectors, the adenovirus system is considered to be an effective vector for introducing exogenous genes and small interfering RNA into all types of cells (dividing and non-dividing) (15). Adenoviruses possess a high transfection efficiency (~100%), low genotoxicity, a marginal possibility of carcinogenic mutation (its genome is not integrated into the host cell genome), and is easy to use with recombinant DNA techniques. In addition, the adenoviral genome is large (36 kb), therefore, the insertion of larger fragments is straightforward. Furthermore, by using adenoviral vectors, a transfection efficiency of 50–90% is more likely without inhibiting DC activity and immune function. However, the adenovirus system is not flawless; the reduced duration of action in transient transfection and the immunogenicity of the adenovirus vector remain to be resolved (16,17).

Thus, the present study aims to construct a pAdxsi-GFP-PSMA adenovirus and develop a novel type of DC vaccine to target PCa, as well as to investigate its therapeutic effect in vitro. This may enable its further application in vivo and provide a novel approach for DC-based immunotherapy for the treatment of PCa.

**Materials and methods**

**Reagents.** Restriction endonuclease was purchased from New England Biolabs (UK) Ltd., Co., (Hitchin, UK); T4 DNA ligase was purchased from JingMei Biological Engineering Co., Ltd. (Shenzhen, China); Alkaline Phosphatase, Calf Intestinal (CIP) enzyme was obtained from Promega Corporation (Madison, WI, USA); Invitrogen Plxf DNA polymerase was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA); dNTP was purchased from Sangon Biotech Co., Ltd. (Shangai, China) and primers were synthesized by Sangon Biotech Co., Ltd. A DNA purification kit (column-type centrifugal), and a DNA gel extraction and purification kit (column-type centrifugal) were purchased from Zweig Las Biotechnology Co., Ltd. (Beijing, China); a 1-kb DNA ladder was purchased from DingGuo Biotech., Co. (Shanghai, China); DNA Marker DL2000 was obtained from Takara Biotechnology Co., Ltd. (Dalian, China); the total protein cleaving enzyme and protease inhibitors were purchased from Beyotime Institute of Biotechnology (Haimen, China); a three-color pre-stained protein marker was purchased from Beyotian Biotechnology Co. (Shanghai, China); rabbit anti-human PSMA monoclonal antibody and rabbit anti-human β-actin polyclonal antibody were purchased from Jiangsu Kangwei Biological Co., Ltd. (Dongtai, China). RPMI-1640 and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Inc. (Gibco); recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), recombinant human interleukin (IL)-4, IL-2 and tumor necrosis factor (TNF) were purchased from Tebaco Biological engineering Co., Ltd. (Xiamen, China). Lymphocyte separation medium was purchased from Haoyang Biotechnology Co., Ltd. (Tianjin, China); phosphate-buffered saline (PBS) was purchased from Boster Biological Technology, Ltd. (Wuhan, China); phycoerythrin (PE)-labeled mouse anti-human CD80 (cat no. 557227) and CD86 (cat. no. 560957)antibodies, peridinin chlorophyll protein (PerCP)-labeled mouse anti-human HLA-DR antibody (Cat. no. 551375) and allo-phycocyanin (APC)-labeled mouse anti-human CD83 antibody (cat. no. 551073) were purchased from BD Pharmingen (San Diego, CA, USA). The Cell Counting Kit-8 (CCK-8) assay kit was purchased from Skylight Biotech Co., Ltd. (Akita, Japan); interferon (IFN)-γ and IL-10 ELISA kits were purchased from Dakewei Biotech Co., Ltd. (Shenzhen, China) and a PE/7AAD apoptosis kit was purchased from KeyGen Biotech. Co., Ltd. (Nanjing, China).
Target gene, vector plasmid and cell strains. A cDNA plasmid for pCMV-SPORT6/PSMA construction was purchased from Mitaka Biotechnology Co., Ltd (Wuhan, China). The shuttle plasmid for pShuttle-CMV-EGFP (Shanghai Jikai Gene Chemical Technology Co. Ltd., Shanghai, China), pAdxsi adenovirus backbone plasmid (Shanghai Jikai Gene Chemical Technology Co. Ltd.), super chemically competent Escherichia coli DH5α and HEK293 cells (Shanghai Jikai Gene Chemical Technology Co. Ltd.) were maintained in the laboratory at China Medical University (Shenyang, China). The PCa cell strains, LNCap, Du145 and 22RV were also preserved in this lab.

Construction and identification of PSMA adenovirus plasmid. Using PSMA as the reference sequence, two primers, containing Sfi restriction sites at the 5'-end, were designed as follows: 5′-AAAAAGGCCGCTGCGGCCACCATGTGGAA TCTCTTCAC-3′ for P1 and 5′-AAAAAGGCACTTTGCGCTTGGCTACTTCA-3′ for P2.

Construction and identification of shuttle plasmid, pShuttle-GFP-PSMA. pCMV-SPORT6/PSMA served as a template, and P1, P2, and the Pfu enzyme (pfx DNA polymerase) were used for polymerase chain reaction (PCR) amplification. The amplified product was processed by SfiI digestion to obtain 2.2-Kb PSMA gene fragments. The adenovirus shuttle plasmid, pShuttle-CMV-EGFP was also digested with SfiI, and agarose gel electrophoresis was performed to retrieve a 5.1-Kb vector DNA. The amplified PSMA gene and retrieved vector DNA fragment were joined using T4 DNA ligase at 5.1‑Kb vector DNA. The amplified product was processed by SfiI digestion to obtain 2.2-Kb PSMA gene fragments. The adenovirus shuttle plasmid, pShuttle-CMV-EGFP was also digested with SfiI, and agarose gel electrophoresis was performed to retrieve any PSMA gene fragments. Additionally, the recombinant vector, pShuttle-GFP-PSMA was sent to Shanghai Shenggong Chemical Technology Co. Ltd. (Shangai, China) for sequencing to ensure the insertion sequence and reading frame were correct.

Construction and identification of recombinant adenovirus plasmid, pAdxsi-GFP-PSMA. The shuttle plasmid, pShuttle-GFP-PSMA and the pAdxsi vector were processed by I-CeuI and I-SceI double digestion. The target gene of the shuttle plasmid was retrieved on gel, while the digested vector was collected using an ethanol precipitation method before the supernatant was collected and the virus was purified using a small quantity plasmid extraction kit, and the recombinant adenovirus plasmid, pAdxsi-GFP-PSMA was obtained.

Extraction, identification and titer determination of recombinant adenovirus plasmid. The recombinant virus supernatant (10 µl) was added to proteinase K (10 mg/ml), incubated at 50°C for 1 h and boiled at 100°C for 5 min. Following centrifugation, 2 µl supernatant served as a template for PCR identification. The HEK293 cells were plated in 96-well plates and the concentrated virus solution was added into the cultured cells at different dilutions. The infected cells were incubated at 37°C for 10 days and the number of wells per line that were exhibiting CPE was recorded under a fluorescence microscope. The ratio of positive wells was calculated. According to the 50% Tissue Culture Infectious Dose (TCID50) formula method, for a 100-µl sample, the titer (T) = 10^(d+0.7) (where d is log10 dilution and s is the sum of the ratio of positive wells at each dilution). In order to convert from TCID50/ml to pfu/ml the following formula was used: T = a x 10^(b x 10^(-0.7) pfu/ml).

Induction of peripheral blood mononuclear cell (PBMC)-derived dendritic cells and determination of the efficiency of adenovirus infection in vitro. Blood cell separator was applied for separation and enrichment of PBMCs obtained from ten healthy volunteers (mean age, 39±5 years; 3 male, 7 female). Blood collection performed at The First Affiliated Hospital, China Medical University (Shenyang, China). Ficoll lymphocyte separation medium was used with density gradient centrifugation for preliminary purification of the mononuclear cells. The cells were rinsed twice with PBS, counted and stained with trypan blue (Shenyang Boermei Reagent Co.) to detect cell viability. The cells were resuspended with RPMI-1640 medium containing 10% FBS at
a density of 2x10⁶/ml and seeded in 25-cm² flasks. The cells were incubated at 37°C in an atmosphere of 5% CO₂ for 2 h, and the non-adherent cells were extracted into another culture flask for CTL induction in vitro. The adherent cells were cultured in RPMI-1640 medium containing 10% FCS, with 100 ng/ml GM-CSF and 50 ng/ml IL-4. Half of the medium was changed every three days and fresh cytokines were added. After six days of induction, 1,000 U/ml TNF-α was added to stimulate DC maturation and the mature DCs were collected on day seven or eight.

**Transfection of the empty viral plasmid, Ad-GFP into immature DCs and detection of transfection efficiency at different MOI.** On day five, immature DCs that were half-adherent were collected and seeded in 24-well plates in 0.2 ml medium containing 10% FBS (5x10³ cells/well). The concentration of Ad-GFP was adjusted to 2x10⁷ pfu/μl. The immature DCs were divided into five groups (three wells per group), and the Ad-GFP virus was added to the DCs in each group at MOI values of 50, 100, 200, 300 and 400. DCs containing the virus were incubated at 37°C in 5% CO₂ for 2 h, during which the culture plates were agitated every 15 min to ensure uniform infection of the virus. Then RPMI-1640 medium containing 10% FCS, 100 ng/ml GM-CSF and 50 ng/ml IL-4 was added to each well and the cells were observed under a fluorescence microscope at 12, 24 and 48 h to detect the expression of GFP at one MOI value. At 48 h, flow cytometry was used to detect the GFP-positive rate at different MOI values, and a PE-7AAD apoptosis and necrosis kit was used to detect the apoptosis rate of the DCs, in order to determine the optimal MOI.

**Changes in DC immune function following infection with the PSMA gene recombinant adenovirus**

**DC and CTL culture in vitro.** The method of DC culture is described above. The non-adherent cells were seeded in an additional flask and differentiated into CTLs in the presence of IL-2.

**Expression of PSMA in recombinant adenovirus-mediated DCs.** On the fifth day, half-adherent immature DCs were collected and made into a single cell suspension. The appropriate quantities of Ad-PSMA and Ad-GFP were added into the above-mentioned DC suspension according to the optimal MOI value, creating two groups of DCs: The Ad-PSMA-DC and Ad-GFP-DC groups. After 48 h of incubation at 37°C in a 5% CO₂ atmosphere, the cells were processed with radioimmunoprecipitation assay lysis buffer and protease inhibitors. The cells were collected and the membrane was washed with Tris-buffered saline with Tween-20 (TBST) and incubated at 4°C overnight. The membrane was washed with TBST, three times for 10 min each time, and the horseradish peroxidase (HRP)-labeled secondary antibody (HRP-goat anti-rabbit IgG) was added and the membrane was incubated at room temperature for 1 h. The membrane was washed with TBST a further three times (10 min each time), and visualized in a dark room by enhanced chemiluminescence.

**Detection of DC phenotype before and after virus infection by direct immunofluorescence.** Fluorescent antibodies were PE-labeled CD80 and CD86, peridinin chlorophyll-labeled HLA-DR and allophycocyanin-labeled CD83. Immature DCs on day 5, mature DCs on day 8 and the cells infected with adenovirus for 48 h (Ad-PSMA-DC and Ad-GFP-DC) were collected. The four types of cell were rinsed twice with PBS, centrifuged at 800 x g for 5 min and the cell density was adjusted to ~1x10⁶/ml. Each type of cell suspension (1 ml) was transferred to four EP tubes and the fluorescent antibodies were added. After 30 min of incubation at 4°C, cells in each tube were rinsed twice with PBS, resuspended with 0.5 ml PBS and transferred into FACS tubes (BD Pharmingen) for phenotype analysis.

**Detection of the IL-12 level in the supernatant of the DC vaccine in each group by ELISA.** On the fifth day of DC culture, the recombinant virus was added to each group according to the optimal MOI value and the DCs were divided into three groups: Ad-PSMA-DC, Ad-GFP-DC and normal DCs. On the sixth day, 1,000 U/ml TNF-α was added to each group for DC maturation stimulation. The supernatant of each group was collected after 48 h of transfection, and the quantity of IL-12 in each group was detected according to the ELISA kit instructions.

**Detection of CTL cytotoxicity to the three PCa cell lines (LNCap, Du145 and 22RV) in the different groups by CCK-8 assay.** On the fifth day of DC culture, a recombinant virus was added to each group according to the optimal MOI value and the DCs were divided into three groups: Ad-PSMA-DC, Ad-GFP-DC and normal DCs. The DCs were incubated at 37°C with 5% CO₂ for 48 h to form stimulation cells. Prior to use, 10 μg/ml mitomycin was added and the cells were incubated at 37°C for 30 min, rinsed with RPMI-1640 incomplete medium (serum free) three times and adjusted to a density of 1x10⁶/ml. The non-adherent mononuclear cells were cultured in RPMI-1640 medium containing 10% FBS and 1,000 U/ml IL-2. Every three days, fresh medium and IL-2 were added to induce CTLs, the cell density was adjusted to 1x10⁶/ml and prepared for reaction cells. The above-mentioned stimulation cells (each group of DCs) and reaction cells (CTLs) were co-cultured at a ratio of 1:10 in complete medium at a density of 2x10⁶/ml for 96 h (37°C, 5% CO₂). In addition, the PCa cells (LNCap, Du145 and 22RV) at the logarithmic growth phase were plated in 96-well plates and cultured for 24 h for cell attachment. The DC-CTL of the three groups and the CTL control were added to the plates containing cancer cells at different effector to target (E/T) ratios (10:1, 20:1 or 40:1) and the mixtures were incubated for another 24 h before the CCK-8 reagent was added (concentration, 20 μl/well). After 4 h of incubation at 37°C, the optical density (OD) value of each well was detected with ELISA and the cytotoxicity of CTLs in each group was calculated according to the following formula: Killing rate (%) = [1-(ODexperimental group-ODeffector cells alone)/ODtarget cell alone] x 100.
Statistical analysis. Data were processed with SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) and expressed as means ± standard deviation. Multiple comparisons of sample means were analyzed by one-way analysis of variance and P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of shuttle plasmid, pShuttle-GFP-PSMA. The recombinant shuttle plasmid, pShuttle-GFP-PSMA and empty vector, pShuttle-CMV-EGFP were digested by SfiI and, by PCR amplification, two fragments of ~2.2 kb (consistent with the size of the inserted fragment) and ~5.1 kb were obtained from the pShuttle-GFP-PSMA plasmid, while only the 5.1-kb fragment (recombinant shuttle vector fragment) was obtained from the empty vector (Fig. 1). The results of DNA sequencing were consistent with the reports obtained from GenBank: PSMA (NC_004463.1; http://www.ncbi.nlm.nih.gov/gene/1054986) and FOLH1 (NC_000011.10; http://www.ncbi.nlm.nih.gov/gene/2346).

Identification of recombinant adenovirus vector, pAdxsi-GFP-PSMA. The recombinant shuttle plasmid,
pShuttle-GFP-PSMA and empty vector, pShuttle-CMV-EGFP were digested by SfiI, and loaded onto 1% agarose gel for electrophoresis. The results are presented in Fig. 2.

Construction and identification of the human recombinant adenovirus carrying the PSMA gene. pAdxsi-GFP-PSMA was linearized with PacI for transfection into HEK293 cells. After one week of culture, when the transfected cell showed obvious vacuole-like changes under a fluorescent microscope, the virus DNA was extracted and amplified by PCR. The emergence of a 2.200-bp target band during electrophoresis indicated successful recombination of the target genes into the adenovirus genome (Fig. 3). Subsequent to identification, the concentrated virus solution was used for re-infection of the HEK293 cells at various concentrations and the virus titer was 2.0x10^{10} pfu/ml.

Efficiency of in vitro transfection of the adenovirus into the DCs. Results of flow cytometry (Fig. 4) demonstrate that the transfection efficiency reached a maximum value (89.84±1.13%) at an MOI value of 200, which was significantly different from that at MOI values of 100, 150, 300 and 400 (P<0.05). In addition, the apoptosis rate of the DCs was significantly lower in the MOI 200 group (6.29±0.14%) when compared with the other groups (P<0.05), indicating a minimum impact on DC vitality at an MOI value of 200. Therefore, the optimal MOI value was determined to be 200. When the MOI value was fixed at 200, the GFP expression level was observed under a fluorescent microscope 12, 24 and 48 h after transfection. The results demonstrated that GFP expression was greatest (up to 85%) 48 h following transfection (Fig. 5A-D).

Changes in immune function of DC after transfection with PSMA gene recombinant adenovirus

Morphology of DCs observed by fluorescence microscopy. The DCs were observed on different days under a fluorescence microscope. On day 1, cells were adherent, small, irregularly shaped (with small pseudopodia and no branch-like protrusions) and showed a uniform distribution of cell colonies. On day 5, the majority of cells became larger, semi-adherent irregular spindle cells that clustered and showed bacteria curtain-like protrusions on the periphery. On the seventh day after Ad-GFP transfection, the cell size increased, the spikes on the cell rim became more apparent with obvious branches, and the cells were star- or spindle-shaped, with obvious nuclei and grew in clusters. All of these features were similar to those of DCs, which had been cultured for seven days under normal conditions (Fig. 6A-D).

Expression levels of PSMA in DCs observed by western blotting. Following transfection (48 h), the positive expression rate of GFP was 85%. Western blot analysis revealed a band at around 110 kd, which is consistent with the size of PSMA proteins; however, no such band was observed in the control group (Fig. 7).

Expression of co-stimulatory molecules in DCs in each group following transfection. Flow cytometry revealed that immature DCs on day 5 expressed a reduced quantity of co-stimulatory and major histocompatibility complex class II (MHC II) molecules (Table I). On day 8, the DCs in the transfection and non-transfection groups all showed features of mature DCs (high expression of the co-stimulatory and MHC II molecules); the expression levels of CD80, CD83, CD86 and HLA-DR were significantly higher in the transfected DCs than in the non-transfected DCs (P<0.05). However, no difference was observed between the Ad-PSMA-DC and Ad-GFP-DC groups (P>0.05). The results demonstrate that transfection of the adenovirus promoted DC maturation and significantly upregulated the expression levels of co-stimulatory molecules.

Detection of IL-12 in the supernatant of each group. Following transfection (48 h), the supernatant of each group was collected, and the quantity of IL-12 in the supernatant was measured by ELISA (Fig. 8). The IL-12 level in the Ad-PSMA-DC (79.51±1.60 pg) and Ad-GFP-DC (69.67±1.43 pg) groups was significantly higher than that in the non-transfection (normal-DC) group (28.88±2.97 pg; P<0.05); however, no significant difference was identified between the two transfected groups (Ad-PSMA-DC and Ad-GFP-DC). These results indicate that adenovirus transfection significantly promotes the ability of DCs to secrete IL-12, regardless of whether the cell carries the PSMA gene.

Cytotoxicity of effector cell (CTL) to target cells (LNCap, Du145 and 22RV). A CCK-8 assay was conducted to detect the OD value of each well and the cytotoxicity of CTL in
each group was calculated based on the following formula: Killing rate (%) = \left[1 - \frac{\text{OD}_{\text{experimental group}}}{\text{OD}_{\text{effector cells alone}}} \right] \times 100. Statistical analysis revealed that CTLs that were stimulated by transfected DCs were associated with a stronger killing rate against all three PCa lines, when compared with CTLs that were stimulated by normal cultured DCs or CTLs.

Table I. Phenotype analysis of DCs in each group on days 5 and 8.

<table>
<thead>
<tr>
<th>Group</th>
<th>CD80</th>
<th>CD83</th>
<th>CD86</th>
<th>HLA-DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5d-DCs</td>
<td>22.95±3.34</td>
<td>18.93±1.65</td>
<td>29.64±0.83</td>
<td>29.17±0.81</td>
</tr>
<tr>
<td>Ad-PSMA-DC</td>
<td>33.29±1.13b</td>
<td>36.02±0.71b</td>
<td>53.30±0.86b</td>
<td>53.00±1.62b</td>
</tr>
<tr>
<td>Ad-GFP-DC</td>
<td>29.68±0.61c</td>
<td>32.62±1.07c</td>
<td>50.70±0.83c</td>
<td>49.29±0.53c</td>
</tr>
<tr>
<td>8d-DCs</td>
<td>28.27±1.04a</td>
<td>28.08±1.16a</td>
<td>41.05±1.33a</td>
<td>46.87±1.12a</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD. *P<0.05 vs. 5d-DC group; b P<0.05, c P<0.01 vs. 8d-DC group. DC, dendritic cell; CD, cluster of differentiation; 5d-DC, immature DCs (day 5); PSMA, prostate-specific membrane antigen; GFP, green fluorescent protein; 8d-DC, mature DCs (day 8).

Figure 4. Dendritic cell apoptosis rate at various MOI as evaluated by flow cytometry. As the MOI increased, the rate of apoptosis also increases. At the value of 200, the apoptotic rate of the cells was the highest. *P<0.05. MOI, multiplicity of infection; GFP, green fluorescent protein.
Cytotoxicity reached a maximum value in all of the groups when the E:T ratio was 40:1. Furthermore, at the same E:T ratio, the killing rate of CTL in the Ad-PSMA-DC-CTL group against the LNCap cells (exhibiting high PSMA expression levels) was significantly higher than that against the Du145 and 22RV cells (P<0.05). In addition, the killing rate in the

Figure 5. Transfection efficiency (reflected by green fluorescent protein expression) at a multiplicity of infection value of 200 at (A) 0, (B) 12, (C) 24 and (D) 48 h. (merged, x100).

Figure 6. Morphology of normal cultured DCs on (A) day 1, (B) day 5 and (C) day 7, and (D) ad-GFP-transfected DCs on day 7 (magnification, x400). DC, dendritic cell; GFP, green fluorescent protein.
Ad-PSMA-DC-CTL group was significantly higher than that in the Ad-GFP-DC-CTL, normal DC-CTL and CTL alone groups (Fig. 9A-C; P<0.05 and Table II). The results demonstrate that DCs transfected with PSMA were more effective in eliciting the differentiation of tumor-specific CTLs, which exert a particularly potent killing effect on PCa cells with high PSMA expression levels, such as LNCaP cells.

Discussion

PSMA is a type II transmembrane glycoprotein, which is secreted by prostate epithelial cells. It is composed of 750 amino acids, and has a relative molecular mass of ~100,000. There are three PSMA domains, comprising the intracellular N-terminal, and the transmembrane and extracellular regions. On the intracellular and extracellular regions, there are a plurality of epitopes that may be combined with a variety of monoclonal antibodies (18). Compared with PSA and PAP, PSMA is regarded as a more sensitive and a more specific tumor marker. Its highly specific expression in PCa tissues has established PSMA as one of the leading targets for use within targeted therapies (19). Based on the above-mentioned information, a replication-defective adenoviral vector containing PSMA gene sequences was constructed for use in the present study.

The Adxsi system, which comprised a shuttle plasmid vector system, a virus backbone plasmid and the packaging cell line, HEK293 was selected. The primary advantage of the Adxsi viral packaging system is that the packaged virus is stable and of a high titer, furthermore, the system may be used in the packaging of cytotoxic genes, as well as genes that influence adenovirus physiological processes, such as infection, duplication and packaging (20). The PSMA gene was amplified by PCR and the pShuttle-EGFP-PSMA was directly ligated into the viral plasmid according to a dual restriction site (I-CeuI and I-Scel) directional cloning method. There are few restriction sites of I-CeuI and I-Scel in the human genome, therefore, they can be used to identify longer gene sequences. Additionally, the application of HEK293 cells for the control of PSMA gene expression has improved the efficacy of virus packaging and the virus titer (21). CsCl density gradient centrifugation was performed for purification of the HEK293 cells, including exemption of defective viral particles, cellular debris and a small quantity of medium components, so as to maximize the reduction of pollution-induced immunological responses (22,23) and to obtain a high titer (2.0x10^10 pfu/ml) PSMA adenovirus with high productivity.

DCs are considered to be the most powerful antigen-presenting cells currently known. They are derived from hematopoietic stem cells, and are widely distributed in tissues and organs, but not in the brain. However, the number of DCs in the human body is limited (<1% in human PBMCs), with cancer patients possessing even fewer DCs, which are functionally deficient, as they are incapable of effective antigen presenting (24). In addition, the low immunogenicity of tumor cells and their lack of specific antigens has consistently presented a major issue during vaccine therapy in the activation of human immune responses (25).

There are three stages involved in the in vitro culture of DCs: i) Amplification, ii) differentiation induction and iii) maturation promotion. In the present study, PBMCs were enriched for the induction of DCs in the presence of IL-4, GM-CSF and TNF-α. These cytokines inhibit the transformation of PBMCs into precursor cells, and also induce the generation and maturation of DCs (26,27). However, due to the immune surveillance effect, and strong antigen uptaking and processing capacity of immature DCs, the
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The present study has demonstrated the validity of this method of constructing DC vaccines that are specific to PCa. As a result of the high expression levels of MHC I and II molecules, co-stimulatory molecules (such as CD80 and CD86), adhesion molecules (such as CD44 and CD54, integrin β1 and integrin β2) and characteristic markers (such as CD83), the predominant role of mature DCs is the induction of immune activation. The mechanism of the strong immune stimulatory capacity of DCs is via an interaction between DCs and heat shock protein receptors (including CD91 and Toll-like receptors) on the DC surface, which triggers a series of reactions, including upregulation of surface factors, increased secretion levels of IL-12 and IFN-γ, promotion of DC maturation and improvement of antigen-presenting efficiency (29,30).

In conclusion, in the current study, DCs induced by the PSMA-adenovirus exhibited significantly higher expression levels of CD80, CD86 and CD83, and a significantly higher secretion level of IL-12, when compared with that of DCs that had not been stimulated. However, no such difference was observed between the DCs induced by the PSMA-adenovirus or the empty virus. Furthermore, among all of the DC vaccines, the PSMA-DC vaccine demonstrated a more obvious cytotoxic effect against all three cancer cell lines (particularly against the LNCap cells) when compared with the other vaccines, including the adenovirus-DC vaccine. This indicated that the PSMA-targeted DC vaccine exerted a marked effect against the PCa cells; however, its ability to stimulate DC differentiation and maturation, and to improve their antigen-presenting

Table II. Killing rate of effector cells (CTLs) against three prostate cancer cell lines.

<table>
<thead>
<tr>
<th>Cell line (E:T)</th>
<th>Killing rate (%)</th>
<th>Killing rate (%)</th>
<th>Killing rate (%)</th>
<th>Killing rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ad-PSMA-DC-CTL</td>
<td>Ad-GFP-DC-CTL</td>
<td>Normal DC-CTL</td>
<td>CTL</td>
</tr>
<tr>
<td>LNCap</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:1</td>
<td>72.18±0.43&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>64.28±1.63</td>
<td>63.96±1.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.90±1.39&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:1</td>
<td>80.87±1.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76.92±0.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.51±0.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.08±0.86&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>40:1</td>
<td>89.76±0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80.97±1.12</td>
<td>78.75±0.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.35±0.97&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DU145</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:1</td>
<td>66.61±0.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63.22±1.86</td>
<td>60.83±1.03</td>
<td>35.81±0.76</td>
</tr>
<tr>
<td>20:1</td>
<td>74.49±1.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.96±0.82</td>
<td>64.73±1.68</td>
<td>40.90±1.10</td>
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<tr>
<td>40:1</td>
<td>80.21±1.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76.66±0.37</td>
<td>73.52±1.29</td>
<td>51.84±2.24</td>
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<td>22RV</td>
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<tr>
<td>10:1</td>
<td>68.16±0.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58.81±0.48</td>
<td>56.34±1.23</td>
<td>29.85±0.38</td>
</tr>
<tr>
<td>20:1</td>
<td>76.15±0.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.52±0.74</td>
<td>71.87±1.75</td>
<td>33.46±1.38</td>
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<tr>
<td>40:1</td>
<td>83.50±0.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.66±0.64</td>
<td>74.50±1.45</td>
<td>37.98±0.49</td>
</tr>
</tbody>
</table>

<sup>a</sup>P<0.05, vs. Ad-GFP-DC-CTL; <sup>b</sup>P<0.01, vs. CTL. E, effector; T, target; CTL, cytotoxic T lymphocyte; PSMA, prostate-specific membrane antigen; DC, dendritic cell; GFP, green fluorescent protein. DC, dendritic cell; PSMA, prostate-specific membrane antigen; GFP, green fluorescent protein; CTL, cytotoxic T lymphocyte.

Figure 9. Cytotoxicity of DC vaccine in each group against cancer cells at different effector-to-target ratios: (A) 10:1; (B) 20:1; and (C) 40:1. 1, AD-PSMA-DC-CTL; 2, AD-GFP-DC-CTL; 3, normal-DC-CTL; 4, CTL. DC, dendritic cell; PSMA, prostate-specific membrane antigen; GFP, green fluorescent protein; CTL, cytotoxic T lymphocyte.
efficiency was dependent on the loaded adenovirus. The above result may have been due to the expression of other co-stimulatory molecules that were not identified in the present study; therefore further studies are required to obtain a comprehensive perspective.

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References