Anti-prostate cancer effects of CTL cells induction by recombinant adenovirus mediated PSCA dendritic cells

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Abstract: Immunotherapy has shown evidence of efficacy in reducing the risk of death among primary and metastatic tumor patients. In this study, we develop an antitumor vaccination through cytotoxic T lymphocytes (CTL) induction by recombinant adenovirus (Ad) mediated prostate stem cell antigen (PSCA) dendritic cells (DC) for prostate cancer therapy. DC was stimulated to maturity through infecting with Ad carrying PSCA cDNA. After that, AdPSCA-DC utilized to induce CTL for prostate cancer therapy. The results revealed that AdPSCA infection improved DC maturation including the change of morphological and the expression of surface markers. AdPSCA infection up-regulated the secretion of interleukin-12, but down-regulated interleukin-10 secretion. CTL displayed high IFN-γ secretion level after AdPSCA-DC induced. In vitro, AdPSCA-DC-CTL markedly inhibited PC-3 cell proliferation related to DC-CTL at the effector: target ratio of 20:1. In vivo, AdPSCA-DC-CTL displayed strong prostate cancer cytotoxicity including inhibiting tumor formation, delaying tumor growth and improving mice survival rate. Pathological examination revealed that the numbers of infiltrating AdPSCA-DC-CTL within mice tumor tissues increased relative to DC-CTL. Through further in-depth investigation, the proposed AdPSCA-DC-CTL could serve as an effective anti-tumor immunity strategy against prostate cancer.

Keywords: Prostate cancer, cytotoxic T lymphocytes (CTL), prostate stem cell antigen (PSCA), dendritic cells (DC), adenovirus

Introduction

Prostate cancer that is one of the most common cancers in male's ranks second in cancer related deaths in Chinese men [1]. Date, the major options of prostate cancer treatment is surgery, chemotherapy and radiotherapy, but prostate cancer patients have not cured effectively [2]. Particularly, the curative treatment development for cancer patients, who has developed recurrent related disease because of conventional therapy failed or who has metastatic disease at diagnosis time, is failure. Lots of cancer therapy strategies are developed, such as photoacoustic therapy, photothermal therapy, immunotherapy, hormone ablation therapy et al [3, 4]. Hormone ablation therapy is a novel strategy and can offer palliation, but the majority of cancer patients eventually progress to refractory disease and further unresponsive to other cancer therapy [5]. Therefore, we need to develop novel and effective approaches for preventing and/or managing prostate cancer patients.

Immunotherapy is a promising method for eradicating tumor. Immunotherapy mechanism is that inducing systemic immunity to specific antigens that express on tumor cells but not normal prostate [6, 7]. Dendritic cells (DC), which are professional antigen presenting cells in the mammalian immune system, play pivotal roles in initiation and regulation of innate and adaptive immune responses [8]. The main function of DC is processing antigens captured in peripheral tissues, presenting them on the cell surface of T lymphocytes, and subsequently initiating T cell or B cell immunity [9]. In recent years, DC has been exploited extensively to develop DC-based immunotherapy to treat various puzzle diseases including virus and cancer. For example, ex vivo-generated DC combined with tumor-associated antigens could be as therapeutic vaccines against cancers such as metastatic melanoma, renal cell carcinoma, and B-cell lymphoma et al [10, 11]. Efficient delivery of antigens to DC, stimulate DC to maturation, and present tumor-specific antigens on
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DC membranes are essential requirements for DC-based immunotherapy [12].

The recent study deepens our understanding of immune mechanism. There has a strong negative correlation relationship between CTL (cytotoxic T lymphocytes) number and cancer cell proliferation [13]. Regulating T-cell activation helps us develop better strategies for cancer therapy. Immunotherapy kernel is that inducing specific CTL responses depend on specific tumor-associated target antigens and effectively deliver antigens [13, 14]. Last several years, lots of prostate cancer specific antigens have been studied, such as prostate stem cell antigen (PSCA), prostate-specific antigen, prostatic acid phosphatase, prostate-specific membrane antigen and six-transmembrane epithelial antigen [15, 16]. These prostate-specific proteins could serve as ideal antigens that help CTL to identify and eliminate prostate cancer cells.

PSCA is an attractive therapeutic vaccine antigen because it is overexpression in prostate cancer, especially in metastatic tissues [17]. Significantly, it is limited expression in other organs and tissues [18]. PSCA having 123-amino acids is an ideal target antigen for this study. The PSCA amino acid sequence analysis shows that PSCA that belong to Ly-6/Thy-1 gene family is a membrane-bound protein [17]. Esophagus, stomach, bladder and prostate low express PSCA, but it overexpresses in 33% of primary prostate tumors and bone metastasis tissue [19]. PSCA expression level positive correlates with increasing tumor stage, grade et al [15]. Therefore PSCA is considered as an ideal target for cancer immunotherapy.

In this paper, we developed recombinant Ad encoding PSCA to infect DC for induction CTL and evaluated the cancer therapy efficacy of vaccine through PC-3 cell line and C57BL/6 mice prostate cancer experimental model. Our results showed that AdPSCA-DC-CTL displayed strong prostate cancer cytotoxicity including inhibiting PC-3 cell proliferation, tumor formation, delaying tumor growth and improving mice survival rate.

Materials and methods

Mouse and cancer cell line

Five weeks-old, pathogen-free, male BALB/c mice purchased from Animal experimental center of China Medical University. Institutional Animal Care and Use Committee guided the process of mice studies. PC-3 cells (human prostate cancer cells) cultured in high glucose DMEM (Dulbecco's modified Eagle medium) (Sigma, USA) added with 10% FBS (fetal bovine serum) (Thermo Fisher Scientific, USA), Nutserum at 5% (BD Biosciences), dihydrotestosterone at 10 nmol/L and insulin at 5 mg/ml (Sigma, USA).

Construction of recombinant adenovirus

The shuttle vector pAdxsi Expression System (Calvino match Biological Technology Co. Ltd., China) was used for loading recombinant PSCA cDNA in Ad vector. PSCA cDNA (coding 95 amino acids) structured into the pShuttle-CMV to obtain pAdxsi-PSCA plasmid. pAdxsi-PSCA plasmid transected into DC using Lipofectamine 2000 (Suzhou Baijishi Biological Technology Co. Ltd. China). The null vector (Adnull) was applied as a control.

Generation of monocyte-derived DC

Peripheral blood mononuclear cells (1-3×10⁸) were collected from volunteers by leukapheresis, and then cultured in tissue culture flask (25 cm²) adherence at a density of 1×10⁵ cells/flask with 5 ml of RPMI-1640 medium (Sigma, USA) containing FBS (10%) and human serum albumin (1%) (Baxter, Deerfield, USA). After incubation 2 h (37°C, 5% CO₂), non-adherent cells were removed by rinsing with PBS (phosphate buffered saline). Then adherent cells were resuspended in clinical grade Cell-Gro DC medium (CellGenix, Freiburg, Germany) containing IL-4 (500 IU/mL) (CellGenix, Freiburg, Germany), 1000 IU/mL GM-CSF (CellGenix, Freiburg, Germany) for incubating 5 d. After that, DCs split into three aliquots. PBS, Ad-null and AdPSCA at MOI (multiplicity of infection) of 200 were added to DC culture. After 2 h incubation, FBS (10%) was added to RPMI 1640 medium, the medium consisted of recombinant GMCSF (1000 IU/ml), IL-4 at 500 IU/ml and 1000 IU/ml TNF-α. After 48 h of incubation, DC was harvested.

Preparation of PSCA-specifically stimulated CTL

Those DCs seeded into 24-well plates (5-6×10⁴ cells/well) after irradiated with 40 Gy, and then
we added non-adherent autologous peripheral blood lymphocytes (1-6×10^6 cells/well) from volunteers. Lymphocytes harvested after 7 days of co-culture, and then we seeded lymphocytes (5-6×10^5 cells/well). After that, we further stimulated those cells using irradiated DC (1-6×10^6 cells/well) for 7 days incubation.

Flow cytometric analysis the marker of DC

Collected DC resuspended in cold PBS. After that, DC then incubated with APC (allophycocyanin)-conjugated mouse anti-human CD83 antibody (Abcam, USA), PE (phycocerythrin)-conjugated mouse anti-human CD80 antibody (abcam, USA), FITC-conjugated mouse anti-human CD86 antibody (abcam, USA), and Percp (peridinin-chlorophyll-protein complex)-conjugated mouse anti-human HLA-DR antibody (abcam, USA). Cells (5-6×10^5) incubated with those fluorescence antibodies at 4°C (in dark, 40 min). Then those DC washed using PBS for three times, and then analyzed using FACScan (BD, USA).

Confocal fluorescence imaging

AdPSCA-DC was plated on confocal dish and cultured (37°C, 5% CO_2) for 12 h. APC-conjugated mouse anti-human CD83 antibody, Percp-conjugated mouse anti-human HLA-DR antibody and DAPI (Beijing Biodee Diagnostic Technology Co. Ltd. China) were co-incubated with the cells for 1 h. After PBS washed, cell image performed using laser scanning confocal microscopy (ZEISS LSM 510 META, Germany). APC was excited at 752 nm and recorded at 720-750 nm; Percp was excited at 488 nm and recorded above 677 nm, DAPI was excited at 340 nm and recorded above 488 nm.

Detection of the level of IL-12 and IL-10 in the AdPSCA-DC supernatant

Optimum MOI200 added to DC after five days of culture, and the corresponding virus added as well. DC transfections divided into 3 groups: AdPSMA-DC, Adnull-DC and DC control groups. After six days of transfection, TNF-α (Shanghai Puxin Biological Technology Co. Ltd. China) (1000 U/mL) was added to stimulate DC to mature; after 6 days of transfection, ELISA was employed to detect the level of IL-12 and IL-10 in the cell culture supernatant of each group.

Detection of IFN-γ

DCs, Adnull-DC and AdPSCA-DC cells supplemented with fresh medium and IL-2 to induce CTL cells every three days. CTL cell concentration was adjusted to 1×10^6 cells/ml. Mixed cultures were prepared for three groups consisting of DC-CTL, Adnull-DC-CTL and AdPSCA-DC-CTL on 96-well plates. The IFN-γ on DC-CTL, Adnull-DC-CTL and AdPSCA-DC-CTL were measured using an ELISPOT assay.

Detection cytotoxicity effects of AdPSCA-DC-CTL

PC-3 prostate cancer cells collected, and then seeded into 48-well plates (5-6×10^4 cells/well) in 2 ml culture medium. CTL (1-6×10^6 cells) was added into 48-well plates at 20:1 of E:T after 4 h static cultivation. Cell mixture was washed using cold PBS after 12 h incubation. Those cells stained with propidium iodide (PI) and annexin V-FITC (Invitrogen, USA), and then using flow cytometry (BD Bioscience, USA) analyzed data. Fluorescent emission of FITC was 515-545 nm and excitation of FITC was at 488 nm, fluorescent emission of DNA-PI complexes was 564-606 nm and excitation was at 488 nm. Compensation was used wherever necessary.

Immunization and tumor challenge

Nude BALB/c mice (male) were intravenous injection of 1×10^6 DC-CTL, Adnull-DC-CTL and AdPSCA-DC-CTL. After one week, mice were subcutaneous injection of 2×10^6 PC-3 cells at vertebral flank. 40 days following last vaccination, tumor volume was calculated using formula V = (L×W^2)/2, where W was shortest dimension and L was longest dimension. For therapeutic effectiveness detection, 2×10^6 PC-3 cells were subcutaneous injection into the vertebral flank. When mice tumor volume developed to 100 mm^3, (1×10^6) DC-CTL (1×10^6), Adnull-DC-CTL (1×10^6) and AdPSCA-DC-CTL (1×10^6) were intravenous injected at once a week. Three weeks after injection, mouse weight, tumor sizes and medium survival times were recorded.

Histological staining

Tumors collected from the euthanized PC-3 cells tumor bearing BALB/c male mice after
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immunotherapy. Tumor tissue was fixed in a formaldehyde (4%) solution (Aladdin, China) for at least 48 h at room temperature. H&E staining (BBC Biochemical, USA) and immunohistochemical staining were performed and then observed with a BX41 bright field microscopy (Olympus, Japan).

Statistical analysis

Statistical analysis was conducted with SPSS 19.0 software. Results were expressed as mean ± SD. Student’s t test was used to assess differences. P < 0.05 represented statistically significant.

Results

Characteristics of DC

DC was isolated from human peripheral blood and activated through infecting with AdPSCA in vitro. DC morphological under a microscope (Figure 1A) displayed typical modifications from small, unequal size and round to large and cell aggregation. After that, we then detected the expression of surface molecule on AdPSCA DC by FACS. The FACS data in Figure 1B showed that the percentage of surface markers CD80, CD83, CD86 and HLA-DR was 39.6%, 55.2%, 62.4% and 67.8%, respectively. Those surface molecules on AdPSCA-DC were further studied using confocal laser scanning microscope (CLSM). Confocal images (Figure 1C) showed CD83 and HLA-DR appeared obviously on AdPSCA-DC surface. Those results illustrated that DCs induced successfully to maturity by AdPSCA.

Immune factors expression level of DC and CTL

IL-10 and IL-12 are significant immunoregulatory cytokine, which have important biological function. We detected the expression levels of IL-10 and IL-12 of DC by ELISA. As shown in Figure 2A and 2B, IL-12 expression level in DC,
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Figure 2. The immune factors expression level of DC and CTL. A. ELISA detection of IL-12 expression level of DC, Adnull-DC and AdPSCA-DC; B. ELISA detection of IL-10 expression level of DC, Adnull-DC and AdPSCA-DC; C. ELISPOT assay the IFN-γ level of CTL, Adnull-DC-CTL and AdPSCA-DC-CTL. Compared with DC, *P < 0.05; Compared with Adnull-DC, #P < 0.05.

Figure 3. Annexin V-FITC/propidium iodide (PI) double staining analysis of CTLs cytotoxic against PC-3 cells.

Figure 4. Anti-tumor protection of DCs, Ad-null-DCs and Ad-PSCA-DCs. Adnull-DC and AdPSCA-DC followed by 65.49 ± 9.31, 68.62 ± 9.83 and 97.37 ± 7.76 (pg/ml) and the IL-10 expression level in DC, Adnull-DC and AdPSCA-DC followed by 322.46 ± 36.88, 333.13 ± 2 5.19 and 257.92 ± 33.63 (pg/ml); Compared with DC, IL-12 in AdPSCA-DC secretion increased 1.49 times, but the secretion of IL-10 in AdPSCA-DC reduced a fifth. Meanwhile, either IL-12 or IL-10 was not significant difference between DCs and Adnull-DC. AdPSCA improved the secretion of IL-12 and inhibited the IL-10 secretion. It suggested that immunological reaction of our study was inclined to the Th1 response.

IFN-γ plays a role in tumor cell growth and apoptosis. IFN-γ in CTL with different stimulation was detected using ELISPOT assay. As showed in Figure 2C, IFN-γ level of CTL stimulated with
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DC, Adnull, DC and AdPSCA-DC were 1.88 ± 0.58, 3.05 ± 0.92 and 10.59 ± 1.38 (μg/L), respectively. AdPSCA-DC-CTL expressed the highest level of IFN-γ compared with other CTL groups.

AdPSCA-DC stimulates CTL cytotoxic activity in vitro

To quantitatively evaluate the tumor therapeutic efficacy of AdPSCA-DC, cell viabilities of PC-3 cells with different treatment protocols were evaluated using annexin V-FITC/PI double staining analysis. The result (Figure 3) illustrated that DC-CTLs, Ad-null-DCs-CD8+CTL and AdPSCA-DC-CTL caused 5.6%, 3.7% and 23.8% of PC-3 cells to lysis, respectively, at 20:1 ratio of E:T. AdPSCA-DC-CTL displayed enhanced killing activity among three different kinds of CTL. The killing efficiency of AdPSCA-DC-CTL was 4.25 fold compared with DC-CTL and 6.4 fold compared with Adnull-DC-CTL. It declared that AdPSCA-DC-CTL had enhanced cytotoxicity and provided an effective alternative cancer treatment modality.

Evaluation of therapeutic effect of AdPSCA-DC-CTL in vivo

To evaluate therapeutic effect of AdPSCA-DC-CTL in mice model, firstly, we investigated the function of AdPSCA-DC-CTL to suppress tumor formation in male nude BALB/c mice. Before subcutaneous injecting PC-3 tumor cells (4×10⁶/mice), mice were injection with DC-CTL, Adnull-DC-CTL and AdPSCA-DC-CTL, respectively. After 22 days, there was not a tumor located in mice on AdPSCA-DC-CTL group. However, tumor developed in DC-CTL group and Adnull-DC-CTL group, the size of tumor was up to 1525.74 ± 28.1 mm³ and 1495.31 ± 28.41 mm³, respectively (Figure 4). Those results declare that AdPSCA-DC-CTL can effectively and markedly prevent the formation of tumors.

We further researched the anticancer activities of AdPSCA-DC-CTL in tumor-bearing mice. When tumor volumes grew up to 100 mm³, prostate tumor bearing mice treated with DC-CTL, Adnull-DC-CTL and AdPSCA-DC-CTL, respectively. Follow-
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After the therapy, we monitored the tumor volumes and body weights, periodically. Those quantitative analysis data (Figure 5A) showed that DC-CTL group and Adnull-DC-CTL group had a similar tumor size that rapidly grew up to 25 folds by day 22. However, mice treated with AdPSCA-DC-CTL for 22 days had a small tumor growth up to 5 folds, it significantly lower than others. Representative photos of tumor after various treatments showed the same result (Figure 5B). The medium survival times of mice that treated with DC-CTLs and Adnull-DC-CTL, respectively, were 22 and 22 days, respectively. Significantly, mice treated with AdPSCA-DC-CTL survived up to 37 days (Figure 5C). One of the immunotherapy side effects is the loss of body weight. In our study, we measured mice body weights of different groups for 22 d, the result in Figure 5D showed that mice body weights of different groups were stable. To further assess the toxicity, the pathological examination was performed by hematoxylin and eosin (H&E) staining. The result (Figure 6) indicates that, compared with DC-CTL group, tumor tissue of AdPSCA-DC-CTL group showed severe edema and vacuolation cells, while unnumbered AdPSCA-DC-CTL appear on the tumor tissues, suggesting a reasonable therapy magic for the application. It declared that our immunotherapy strategy had low toxicity. We further examined the footprint of AdPSCA-DC-CTL by immunohistochemistry for making sure whether AdPSCA-DC-CTL could effectively permeate into tumor microenvironment. As show as in Figure 6B, immunohistochemistry stained images revealed vast of infiltrating AdPSCA-DC-CTL within tumor tissues relative to DC-CTL. These data declared that the strong expansion of tumor-specific AdPSCA-DC-CTL correlates to the inhibition of tumor growth by immunization.

Discussion

To develop antitumor vaccine, DC was collected from the peripheral blood of human, and further infected with recombinant Ad encoding PSCA. Ad is chosen as the main candidate for delivering target genes. It can integrate cDNA with genome of host cell by integrase for persistently expressing PSCA protein [20, 21]. It is beneficial to DC maturation and activation. Importantly, Ad is a safe vector that doesn’t
induce side-effects including cancer, immunological rejection and inflammation [22, 23]. DC matured always with CD80, DC83, CD86, and HLA-DR high protein expression [24]. At molecular levels, compared with DC and Adnull-DC, we found that AdPSCA-DC expressed highly level of cell surface marker. The cell surface marker is conductive to the interaction of DCs and native T lymphocyte, it help native T lymphocyte differentiation and maturation. Morphologically, the AdPSCA-DC showed typical size and shape changes, transfection with Ad could also enhance DC maturation. The mechanism may be that AdPSCA entry into DC and/or translocation to DCs nuclei. Ad capsid protein penton activated NF-κB to up-regulate a lot of protein expression such as immune response, TNF-α, an immune response protein, improves DC maturation through an autocrine pathway [25, 26]. DC is the strongest professional antigen-presenting cell. PSCA disassembled to peptide in DC and then connected with MHC-I before transporting to cellular membrane for T cell antigen receptor recognition [27].

Activated DC can secrete various cytokines, such as IL-12 and IL-10 [28]. In our study, AdPSCA-DC possessed the character of the highest expression level of IL-12 and the lowest expression level of IL-10. High expression IL-12 could initiate protective innate and adaptive immune responses against cancer cell [29]. In immunological reaction, IL-12 can be beneficial to develop Th1 type reaction that induces the IFN-γ expression for killing tumor cells [30]. However, IL-10 as an inhibitory factor initiates Th1 type reaction and T lymphocyte. AdPSCA infection inhibits IL-10 secretion that up regulates the expression level of IFN-γ, TNF-α and GM-CSF [31-33]. Inhibit IL-10 secretion enhances antigen-presenting ability and inhibits immnity escape of carcinoma [34].

Antigen-specific CTL is a promising immunotherapeutic cell against cancers. T-cell receptor (TCR) of native T lymphocyte recognizes specific major histocompatibility complex (MHC)-antigen complexes along with co-stimulatory molecules [35]. It initiates related signaling pathway and promotes native T lymphocyte differentiate into CD8+CTL and CD4+CTL. Simultaneously, the proliferation capacity of CTL enhanced. In this study, we found that CTL was induced successfully by AdPSCA infected DC. Cell-mediated immunity based on CD8+CTLs plays a primary role in cancer therapy. Nevertheless, the mutual recognition of TCR on native T lymphocytes and tumor antigen presented by DC is under the influence of cancer cells killing efficiency [36]. PSCA up-regulates in some cancers surface including prostate cancer, bladder cancer and pancreatic cancers [17, 37]. The PSCA expression level positively correlated with prostate cancer metastasis, advanced clinical stage and malignant progression of premalignant prostate lesions [38]. Therefore, PSCA was chose as an ideal target antigen. AdPSCA-DC-CTL had strong cytotoxic activity against PC-3 cell which highly expressed PSCA. IFN-γ expression also confirmed that CTL stimulated by AdPSCA-DC had remarkable cytotoxic activity. IFN-γ can activate NK cells, and it is a marker for activating Th1 cells [39]. IFN-γ activates JAK-STAT pathway through IFN-γ receptor for killing cancer cells [40]. AdPSCA-DC-CTL was useful and could further develop for prostate cancer immunotherapy in vivo.

We further perform in vivo animal experiment to verify our current data. AdPSCA-DC-CTL vaccination suppressed exogenous PC-3 prostate cancer cell to format tumor. Importantly, AdPSCA-DC-CTL delayed the development of xenograft tumor. Immunohistochemical staining showed that lots of AdPSCA-DC-CTL appeared in tumor tissue, it was beneficial to damage tumor cell. There are two ways for AdPSCA-DC-CTL to kill tumor cells. One is the secretion of perforins and granymes [41]. In the presence of Ca2+, perforin can be inserted into tumor cell membranes, and polymerization forming tubular structure to destroy the structure of the target cell membranes, while granymes as a kind of serine esterases, enter the cytoplasm to directly activate protease in cytoplasm leading to the cell apoptosis [42, 43]. On the other hand, high level FasL on CTL and Fas on the surface of tumor cells mutual recognized to make the tumor cell programmed cell death [44, 45].

Conclusion

DCs that infected with recombinant Ad encoding PSCA specifically activated cytotoxic T lymphocytes against prostate cancer. AdPSCA-DC-CTL markedly inhibited PC-3 cell proliferation. In vivo, AdPSCA-DC-CTL displayed strong prostate cancer cytotoxicity including inhibiting tumor formation, delaying tumor growth and improv-
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ing mice survival rate. It provides a novel strategy for immunotherapy of prostate cancers.

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Disclosure of conflict of interest

None.

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