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Original Article

Mycobacterial Ag85A-Ag85B double gene adenovirus inhibits lewis lung cancer growth in mice

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Abstract: Background: Immunotherapy is the most promising novel treatment for cancer and expected to achieve breakthrough in near future because of improvement in the understanding of the tumor immunology. The present study is aimed to pave way to explore possibility and approaches of active immunotherapy for lung cancer based on DNA vaccines containing antigen genes of mycobacterial Ag85A and Ag85B. Methods: Mycobacterial antigen genes adenovirus Ad-Ag85A-Ag85B-CMV was constructed. Lewis lung cancer (LLC) cells were infected with Ad-Ag85A-Ag85B-CMV and then Ag85A and Ag85 expression was detected with Western blot. Splenocytes of C57BL/6 mice that were vaccinated with Bacillus Calmette-Guerin (BCG) 6 weeks before were separated and co-cultured with Ad-Ag85A-Ag85B-CMV infected LLC cells. Interleukin-2 (IL-2) and interferon-γ (IFN-γ) in co-culture medium was detected by enzyme-linked immunosorbent assay (ELISA). LLC bearing mice were developed with C57BL/6 mice vaccinated with BCG 6 weeks before. Then, Ad-Ag85A-Ag85B-CMV was intra-tumorally injected so that its inhibitory effect for LLC in mice could be observed. Results: Mycobacterial antigens of Ag85A and Ag85B were detected in Ad-Ag85A-Ag85B-CMV transfected LLC cells with Western blot. An elevation of IL-2 and IFN-γ in co-culture medium was detected by ELISA, compared with empty adenovius and blank control. Average weight of tumors in mice of BCG vaccination and Ad-Ag85A-Ag85B-CMV treatment group was significantly lower than control groups. Conclusion: Immune gene-therapy using mycobacterial antigen genes of Ag85A and Ag85B is promising novel treatment for lung cancer.

Keywords: Lung cancer, immunotherapy, mycobacterial antigen, Ag85 complex

Introduction

Attribute to low-dose CT screening, more lung cancer patients are diagnosed at early stage and cured through surgical procedure [1]. Identification of epidermal growth factor receptor (EGFR) mutations and EGFR tyrosine kinase inhibitors (EGFR TKIs) treatment has also improved prognosis in selected subgroups of patients with advanced stage non-small cell lung cancer (NSCLC) during the past decade [2, 3]. However, long term outcomes of most advanced stage lung cancer patients still remain dismal, and about 30-40% of patients pathologically diagnosed as early stage lung cancer eventually die from local relapse and distant metastasis after surgical resection [4, 5]. Novel effective therapeutic approaches are badly needed to change present status of lung cancer treatment. Immunotherapy is the most promising novel treatment for lung cancer and expected to achieve breakthrough in near future because of improvement in the understanding of the tumor immunology [6, 7].

According to immuno-editing theory, immune response of host against tumor experience 3 phases: elimination, equilibrium and escape. During elimination phase, immune response of host is activated to kill tumor cells mainly through cytotoxic mechanisms. However, small part of the tumor cells can escape from elimination and enter next phrase: equilibrium phase. These tumor cells have a non-immunogenic phenotype and can grow under selection of immune response of host. This process is similar to Darwinian selection and usually lasts for many years. During equilibrium phase, tumor cells get further resistance to immune response of host because of various genomic

mutations. All the cancer patients in clinical practice are in last phase of immuno-editing: escape phase. During this phase, tumor cells escape from immuno-surveillance of host immune system through different mechanisms and usually quickly proliferate and cause local invasion, or even distal metastasis. So, cancer immunotherapy should be based on immune escape mechanisms [8]. Cancer immunotherapy is to provoke the immune response of host, including cellular immunity and humoral immunity, to inhibit or even kill tumor cells [9, 10]. There are different strategies of immunotherapy for cancer, one of which is transduction of immuno-stimulatory xenogeneic genes into tumor cells to enhance immunogenicity of cancer cell, so that stronger immune response of host against tumor cells could be activated [11, 12].

In present study, we constructed adenoviral vector containing mycobacterial antigen genes of Ag85A and Ag85B. Then, we investigated if Ag85A-Ag85B adenoviral vector infected murine Lewis lung cancer (LLC) cells could activate splenocytes of BCG vaccinated mice *in vitro*. And we investigated therapeutic effect of combination of BCG vaccination and Ag85A-Ag85B adenoviral vector injection in LLC tumor bearing mice. The present study is aimed to pave way to explore possibility and approach of an active immunotherapy for lung cancer based on DNA vaccines containing the genes of mycobacterial Ag85A and Ag85B.

Materials and methods

Cell culture and animals

LLC cells and NIH3T3 mouse fibroblasts were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. C57BL/6 female mice (4-5 weeks old) were purchased from Experimental Animal Center of Sichuan University (Chengdu, China) and were fed in special pathogen-free (SPF) conditions in Experimental Animal Center of Luzhou Medical College.

Construction of recombinant adenoviral vectors

The recombinant adenovirus vector containing double gene Ag85A and Ag85B driven by CMV promoter was constructed with Gateway™ clone technology. Briefly, Mycobacterial antigen gene Ag85A and Ag85B cDNA connected with P2A sequence (Ag85A-MYC-P2A-Ag85B-HA) were chemically synthesized, in which Myc and HA were tag sequences connected to Ag85A and Ag85B genes. Then, Ag85A-MYC-P2A-Ag85B-HA sequence was cloned into the adenoviral shuttle plasmid pAdeno-mCMV-EGFP-3FLAG. Then, adenoviral genomic plasmid of AdMax™ system (Microbix Biosystems Inc, Canada) and shuttle plasmid were co-transfected into 293 cells, so that Ag85A-Ag85B expression adenovirus driven by CMV promoter (Ad-Ag85A-Ag85B-CMV) was packaged.

Western blot analysis

The cells were collected and washed twice with 4°C Phosphate Buffered Saline (PBS), then lysed with 100 μl 2× lysis buffers, and total proteins were extracted with routine approach. Concentration of proteins was quantitated using a bicinchoninic acid (BCA) assay kit. 50 μg proteins of each sample were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred onto PVDF membrane (GE Healthcare, Buckinghamshire, UK). Non-specific binding sites were blocked with 5% non-fat milk in Tris-buffered saline-Tween-20 (TBST) for 1 h at room temperature. Then, the samples were incubated with primary anti-Myc or anti-HA antibody (TianGen Biotech, Beijing, China) over-night at 4°C, at concentration of 1:1000. Membranes were washed 10 min for three times in TBST, and then incubated with secondary goat anti-mouse antibody (Santa-Cruz) for 2 h at room temperature. Reactive bands were detected with ECL+ plusTM Western blotting system (Amersham Pharmacia Biotech, Freiburg, Germany). β-actin was used as internal control detected using monoclonal anti-β-actin (Beyotime Biotech, China).

Isolation and culturing of the splenocytes

A 5-week C57BL/6 female mouse was inoculated with 2.5×10^4 CFU of BCG suspended in 200 μl PBS, subcutaneously injected in abdomen. Six weeks later, the mouse was sacrificed by cervical displacement and immediately immersed in 75% ethanol for 5 minutes. The spleen was aseptically taken out and minced well, then pass through 300-mesh stainless steel cell sieve. The suspension was collected

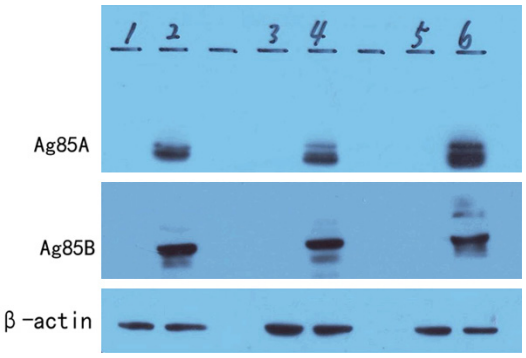

Figure 1. Expression of Ag85A and Ag85B was detected in Ad-Ag85A-Ag85B-CMV infected LLC, A549 and TE-1 cells by Western blot analysis. Lane 1, 3, 5 are control groups; lane 2: LLC cells, lane 4: A549 cells, lane 6: ET-1 cells.

and washed with D-Hank's solution, then 1000 RPM centrifuged for 5 minutes. The pellet was re-suspended with ACK lysing buffer for erythrocyte lysis. Then, the cell suspension was 1000 RPM centrifuged for 5 min, and the pellet was re-suspended with RPMI-1640 medium with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2.

Preparation of stimulator cells

LLC cells were seeded in culture plates and infected with adenoviral vector Ad-Ag85A-Ag85B-CMV 24 hours later, while empty adenoviral vector was used as negative control. Forty-eight hours after infection, cells were treated with 5 μg/ml Mitomycin C for 2 hours.

Proliferation and activation assay of spleen lymphocytes

C57BL/6 mice were vaccinated with BCG. Six weeks later, splenocytes were isolated and were co-cultured with same number aforementioned stimulator cells for 3 days. Then, the culture medium was collected for interleukin-2 (IL-2) and interferon-γ (γ-INF) detection with enzyme-linked immunosorbent assay (ELISA).

BCG vaccination and Xenograft tumor in vivo

Twenty four mice were randomly and equally divided into four groups, so that six mice per group. BCG was subcutaneously injected in abdomen of mice in 3 groups, 2.5×10^4 CFU suspended in 200 μl PBS for each mouse. Normal saline (NS) of same volume was injected in mice in the forth group as control. Six weeks later, all the mice in four groups were subcutaneously injected with LLC cells in left flank near scapula region, 2×10^6 LLC cells in 100 μl PBS per mouse. When tumors in mice were visible, calipers were used to measure larger diameter and smaller diameter of tumors every 2 days, and the volume of tumors were calculated according to the formula: V (mm^3) = larger diameter (mm) × smaller diameter^2 (mm^2) × π/6. When volume of xenograft tumors in most mice reached about 50 mm^3 (the 10th day in present research), mice were intra-tumorally injected with recombinant adenoviral vectors Ad-Ag85A-Ag85B-CMV, 1×10^9 pfu in 100 μl PBS per mouse. Empty vector of same amount were used as negative control. And the adenoviral vector injection was repeated every 3 days, totally 3 times. The mice were sacrificed by cervical dislocation at 10th day from first adenoviral injection. Tumors in all the mice were integrally excised and accurately weighed with electronic balance.

Statistical analysis

All the results are expressed as the means ± standard deviation. Statistical significance was determined using SPSS 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was performed for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Detection of Ag85A and Ag85B in recombinant adenovirus Ad-Ag85A-Ag85B-CMV infected LLC cells by Western blot

Western blot was used to find if Ag85A and Ag85B genes transduced through adenovirus infection could express in different mice cancer LLC cells and of and human lung cancer A549 cells, human esophageal cancer TE-1 cells. As shown in **Figure 1**, both Ag85A and Ag85B were detected in all the kinds of cancer cells.

Cytokines production of mice splenocytes was induced by co-culture with Ad-Ag85A-Ag85B-CMV infected LLC cells

Enzyme-linked immunosorbent assay revealed that IL-2 production of splenocytes of BCG vaccinated mice was significantly elevated by co-culture with Ad-Ag85A-Ag85B-CMV infected

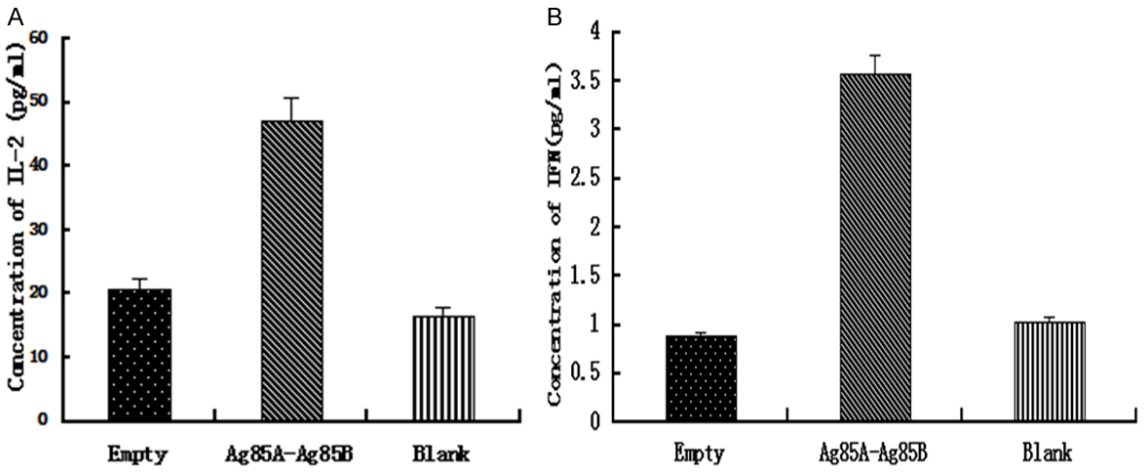

Figure 2. Cytokines production by mice splenocytes co-cultured with Ad-Ag85A-Ag85B-CMV infected LLC cells. A. IL-2 level in medium of mice splenocytes co-cultured with Ad-Ag85A-Ag85B-CMV infected LLC cells was significantly higher than control groups. B. IFN-γ level in medium of mice splenocytes co-cultured with Ad-Ag85A-Ag85B-CMV infected LLC cells was significantly higher than control groups.

LLC cells, compared with empty control (empty adenvirus infection) and blank control ($P< 0.001$, $F=115$). The similar phenomenon was observed in γ-INF ELISA assay ($P<0.001$, $F=404$) (**Figure 2**).

Therapeutic effect of Ad-Ag85A-Ag85B-CMV on LLC tumors in mice xenotransplantation model

We studied the therapeutic effect of Ad-Ag85A-Ag85B-CMV on growth of LLC cells in mice. Six weeks after BCG vaccination, mice were inoculated with LLC cells. One week after inoculation of LLC cells, when tumor volume in most mice reached 50 to 100 mm^3, Ad-Ag85A-Ag85B-CMV or empty adenovirus was intratumorally injected. At end of the treatment experiment, average weight of tumors in BCG+Ad-Ag85A-Ag85B-CMV treatment group was significantly lower than control groups ($F=3.756$, $P=0.027$) (**Figure 3A**, **3B**).

Discussion

Tumor antigen is the most important factor of immunogenicity of tumor cells. However, tumor cells derive from host's own cells due to genetic and epigenetic variations, so immunogenicity of tumor associated antigens TAAs (self antigens) is too weak to initiate effective immune response of host [13]. Xenogeneic antigen activates much more fierce immune reaction, just like in bacterial infection. And this fierce immune reaction is helpful and effective in elimination of microbies. Theoretically, expression of xenogeneic antigens by tumor cells can increase their immunogeicities and provoke strong immune responses that are able to eliminate tumor cells. Several researches reported recent years confirmed possibility and feasibility of this theory, although there is a very long way from bench to clinical practice [14, 15]. The Ag85 complex is a 30-32 kDa family of three proteins named as Ag85A, Ag85B, and Ag85C. Ag85 complex possess enzymatic mycolyl-transferase activity and play important role in composition of cell wall. The three members of Ag85 complex exist in all mycobacteria. Among of them, Ag85A and Ag85B are major immunodominant antigens of M. tuberculosis. They can induce strong T helper type 1 (Th1) immune responses in situations of controlled mycobacterial infection, characterized by proliferation of CD4+ and CD8+ T cells, and increased production of cytokines of interleukin 2 (IL-2) and interferon-γ (IFN-γ) [16]. Druing the past 2 decades, various DNA vaccine against Tubercolosis encoding Ag85A, Ag85B or/and other components of M. tuberculosis antigens were developed. Some of the novel DNA vaccines showed better protection against TB than traditionally used BCG vaccine in animal experiments, or in clinical trials [17, 18]. Even more, it was reported that a novel DNA vaccine expressing Ag85A-HA2 fusion protein could provide protection against influenza A virus and Staphylococcus aureus [19].

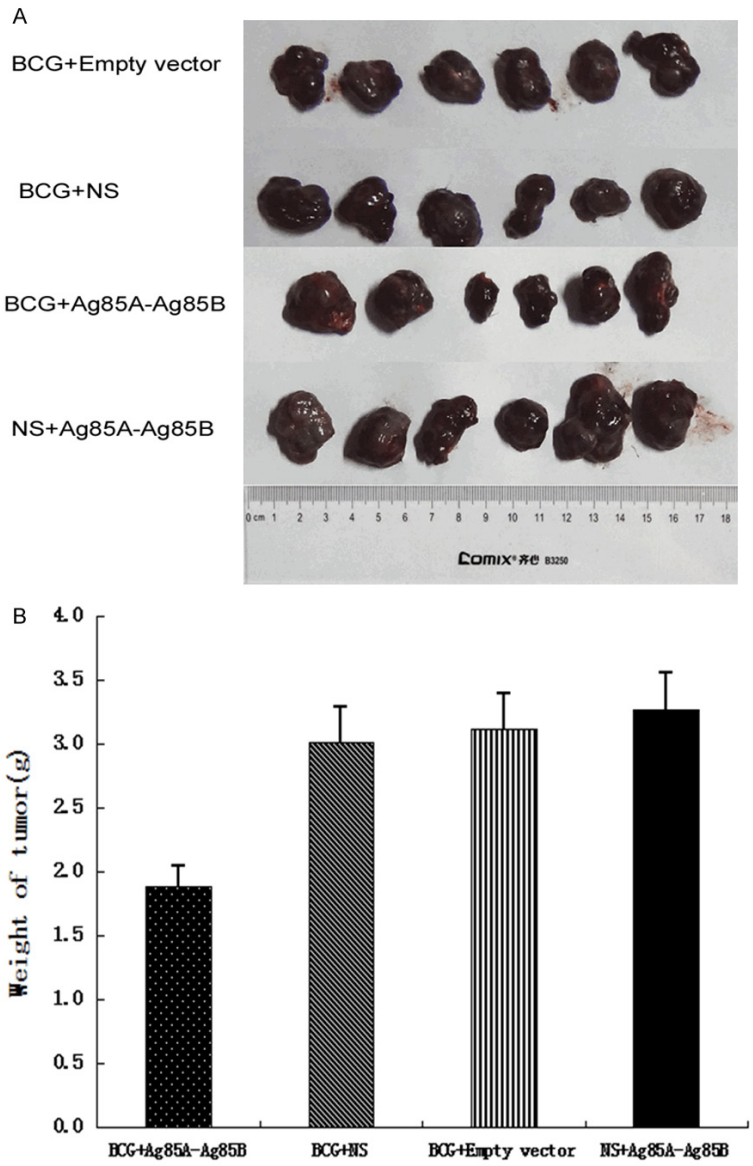

Figure 3. Anti-tumor effect of BCG+Ad-Ag85A-Ag85B-CMV treatment on LLC tumor growth in vivo. A. The tumors of subcutaneous xenograft in different groups. B. Comparison of average tumor weight among different groups. Average weight of tumors in BCG+Ad-Ag85A-Ag85B-CMV treatment group was significantly lower than other groups.

BCG has been used in immunotherapy for bladder cancer since about 40 years ago. Researches revealed that BCG infection of bladder tumor cells could increase expression of antigen-presenting molecules, and subsequently induce immune response via cytokine release, including IL-2, tumor necrosis factor (TNF) and IFN-γ [20]. This complex immune response finally induces anti-tumor activities mediated by cytotoxic T lymphocytes, natural killer cells and macrophages. Recent years, researchers developed DNA vaccine encoding Ag85A, Ag-85B and other M. tuberculosis antigens, trying to improve effect of immuotherapy for bladder cancer while minimize side effect of BCG treatment [21]. Zhang et al developed an Ag85A engineered dendrite cells (DCs) vaccine and found it exerted enhanced anti-tumor immunity against bladder cancer [22].

In clinical practice, individual can get immune memory through initial TB infection or BCG vaccination. When he or she got TB infection for the second time, fierce inflammatory reaction occur at infection site, even ulcer forms, but extensive dissemination of TB seldom occur. This is so called Koch's reaction. The present study is aiming to explore the possibility and feasibility to utilize this immune mechanism into treatment for lung cancer. We postulate host could develop immune memory to some immunostimulatory antigenes of TB after BCG vaccination. If tumor cells in host could produce some immunostimulatory antigenes of TB, fierce immune reaction would be induced in and around tumor. So that tumor cells could be eliminated by fierce immune reaction without severe impairment to normal structures. Based on aforementioned assumption, we constructed adenovirus containing cDNAs of immunostimulatory TB antigen genes: Ag85A and Ag85B. Then, it was confirmed by Western blot that Ag85A and Ag85B could express in LLC cells infected with Ag85A-Ag85B adenovirus. We vaccinated mice with BCG in order to induce immune memory against TB in mice immune system. Six weeks after BCG vaccination, mice splenocytes were separated and co-cultured with Ag85A-Ag85B adenovirus infected LLC cells. An elevation of IL-2 and interferon-γ

(IFN-γ) in co-culture medium was detected by ELISA, compared with empty adenovius and blank control. IL-2 and IFN-γ are important cytokines that play key roles in immune response. IL-2 promotes differentiation of T cells mainly produced by natural killer (NK), CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) when antigen-specific immunity develops [23]. It's also an important activator of macrophages and inducer of Class I major histocompatibility complex (MHC) molecule expression [24]. This finding suggested that Ag85A and Ag85B expression by LLC cells could activate immune cells, especially T cells *in vitro*.

In present research, animal experiments were performed to investigate therapeutic effect of Ag85A-Ag85B adenovirus on LLC tumor burdening mice. In order to enhance immune memory of mice immune system, BCG vaccination was performed 6 weeks before LLC cells inoculation. Present research revealed no impact of BCG vaccination on tumorgenicity of LLC in mice. Then, BCG vaccinated LLC tumor bearing mice were intra-tumorally injected with Ag85A-Ag85B adenovirus. A significant inhibition of tumors was observed in mice treated with Ag85A-Ag85B adenovirus, compared with mice in blank control group and empty adenovirus treated group.

In general, our research showed that Ag85A and Ag85B gene transfected LLC cells could induce immune response of mice, and growth of LLC tumor in mice could be inhibited by Ag85A-Ag85B adenovirus intratumoral injection, although further research is needed to reveal the accurate mechanism. Immune gene-therapy based on Ag85A-Ag85B for lung cancer is a novel and promising but challenging treatment for lung cancer.

In summary, our study revealed that transduction of Mycobacterial antigens genes of Ag85A and Ag85B into LLC cells could enhance their immunogenicity. BCG vaccination combined with Ag85A and Ag85B adenovirus injection could inhibit growth of LLC cells in mice. Immune gene-therapy using mycobacterial antigen genes of Ag85A and Ag85B is promising novel treatment for lung cancer.

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Disclosure of conflict of interest

None.

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