Combination antitumor effect of GM-CSF tumor vaccine with cisplatin for mice with subcutaneous transplanted tumor

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Abstract: Background: Non-small-cell lung cancer has a relatively high remission rate for initial chemotherapy. However, it is very liable to appear adoptive resistance. Irradiated tumor cells modified with GM-CSF are very effective in triggering immune responses. Therefore, combined therapy of tumor cell vaccine expressing GM-CSF with chemotherapy for NSCLC should be focused on. Objectives: To study the effect of combined Lewis lung cancer (LLC) cell vaccine expressing GM-CSF with cisplatin on subcutaneous transplanted tumor mice. Methods: Growth inhibitory rate of LLC cells transfected with Ad5-GM-CSF and Ad-control were evaluated with the method of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay in vitro. Tumor volume and survival were observed for subcutaneous transplanted tumor mice treated with LLC cell vaccine expressing GM-CSF or/and cisplatin, and pathological examination and western blotting analysis were performed for evaluating LLC cell proliferation and apoptosis. Results: LLC cells transfected with recombinant adenovirus containing GM-CSF had a dramatically increased growth inhibitory rate. LLC cell vaccine expressing GM-CSF could significantly inhibit tumor growth and prolong survival of subcutaneous transplanted tumor mice, and combination of this vaccine with cisplatin could further inhibit tumor growth and prolong survival of subcutaneous transplanted tumor mice. LLC cell vaccine expressing GM-CSF could significantly increase the apoptosis index of cells in tumor tissue, and combination of this vaccine with cisplatin could further increase the apoptosis index. Conclusions: Combined therapy of LLC cell vaccine expressing GM-CSF with cisplatin had an enhanced antitumor effect for subcutaneous transplanted tumor mice.

Keywords: Lewis lung cancer, GM-CSF, cisplatin, subcutaneous transplanted tumor, antitumor effect

Introduction

Lung cancer is one of the leading causes of cancer-associated mortality worldwide [1-3]. As the commonest type of lung cancer, non-small-cell lung cancer (NSCLC) accounts for 85% of all lung cancers and has a 5-year survival rate of 4% in metastatic cases [4]. NSCLC has a high malignancy, and its metastasis is early and extensive. NSCLC is sensitive to chemotherapy and radiotherapy and frequently treated with systemic chemotherapy [5]. NSCLC has a relatively high remission rate for initial chemotherapy. However, it is very liable to appear adoptive resistance. Therefore, there is an urgent requirement for new and effective therapies [6].

As a potential cytokine activator of antigen presenting cells (APCs), GM-CSF has an important role in the development of antitumor immune responses and breaking tolerance [7]. The irradiated tumor cells modified with GM-CSF are very effective in triggering immune responses [8]. Therefore, GM-CSF is frequently used as the adjutant of tumor vaccine [9]. Cisplatin is a platinum coordination compound, which is the first platinum compound for cancer treatment with the approval of Food and Drug Administration (FDA) [10]. Cisplatin is frequently employed in the chemotherapy of NSCLC. Recently, combined therapy of cisplatin with other drugs has been applied in many human cancers to reduce drug resistance and alleviate side effects [11]. In this paper, NSCLC tumor cell vaccine modified with GM-CSF was firstly prepared, and the effect of combined this vaccine with cisplatin on subcutaneous transplanted tumor mice was then evaluated. The aim is to provide
a basis for combined therapy of NSCLC tumor cell vaccine expressing GM-CSF with cisplatin.

Materials and methods

Mice

Male 7-week-old C57BL/6 mice were purchased from the Laboratory Animal Center of Xinjiang Medical University (Urumqi, China) and kept under specific pathogen-free conditions in the Laboratory of Biotherapy of Affiliated Tumor Hospital of Xinjiang Medical University (Urumqi, China).

Experimental research performed in the paper has been approved by the Animal Care and Welfare Committee of Affiliated Tumor Hospital of Xinjiang Medical University (approval number: 20130009). All experimental research on mice followed the Guide for the Care and Use of Laboratory Animals (8th edition, 2011).

Mouse Lewis lung cancer cell line

Mouse Lewis lung cancer cell line was cultured in DMEM medium containing 10% FBS (Gibco-BRL, Gaithersburg, MD, USA), and NK (GK1.5, ATCC) in IMEM medium containing 20% FBS (Gibco-BRL, Gaithersburg, MD, USA). All mediums were supplemented with 20 mM L-glutamine, 100 μg/ml of streptomycin and 100 U/ml of penicillin. Tumor cells were kept at 37°C in a humidified atmosphere containing 5% CO₂. Cell transfection was performed with Cationic liposome DOTAP-Chol following the manufacturer’s standard procedure [12].

Vaccine preparation

The coding sequence of mouse GM-CSF gene (33-458, gi:51100) was determined through retrieval in Gen Bank. Primers with NotI and HindIII restriction enzyme cutting sites were designed with Oligo 6.0 software (Molecular Biology Insights, Inc. USA). Forward primer: GM-CSF-Not-I-447-F5’-ATTTGCGGCCGCATGTGGCTGCAGAATTTACTTTTCC-3’, reverse primer: GM-CSF-HindIII-447-R5’-GCCAAGCTTCTATTTTCTATTTTCTATTTTCTATTTTCTATTTTCTATTTTCTATTTTCTATTTTCTATTTTCTATTTTCTAT TT-3’. The length of the PCR products was 438 bps. MFL15673-GM-CSF was used to amplify GM-CSF gene as a template. The PCR products were recycled and then digested with NotI and HindIII, and pDC316-CMV-EGFP was also digested with NotI and HindIII. The both restriction fragments were then connected with T4 ligase for shuttle plasmid pDC316-CMV-EGFP-GM-CSF. Shuttle plasmid pDC316-CMV-EGFP-GM-CSF was then greatly prepared after the integration of GM-CSF gene into pDC316-CMV-EGFP plasmid was verified through enzyme digestion and sequencing analysis. Recombinant adenovirus packaging cells HEK293 were co-transfected with pDC316-CMV-EGFP-GM-CSF and skeleton adenovirus plasmid pBH Glox(delta)EI, 3Cre for recombinant adenovirus Ad5-GM-CSF-EGFP, and co-transfected with pDC316-CMV-EGFP and pBH Glox(delta)EI, 3Cre for recombinant adenovirus Ad as a control (Ad-control). Ad5-GM-CSF-EGFP was amplified and purified after detecting green fluorescent protein and PCR analysis. Mouse Lewis lung cancer (LLC) cells were transfected with Ad5-GM-CSF, and the secretion of GM-CSF was then measured. LLC cells reaching to the criterion of the secretion were irradiated with a lethal dose X-ray (100cGy^{60}CO).

Cell viability assays

The single-cell suspension of LLC cells, LLC cells transfected with Ad5-GM-CSF and Ad-control was prepared. Growth inhibitory rates (GIR) of LLC cells transfected with Ad5-GM-CSF and Ad-control were evaluated with the method of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay [13]. The formula for calculating GIR was GIR = [1 - (OD of LLC cells transfected/OD of LLC cells untransfected)] × 100%. Cells were plated in 96-well plates, and the absorbance was measured with a 96-well spectrophotometer using the wavelength of 570 nm at 0 h, 24 h, 48 h, 72 h and 96 h. Each suspension was carried out in 15 replicates at different time points.

Preparation of mouse subcutaneous transplanted tumor model and grouping

Mouse subcutaneous transplanted tumor model was prepared through subcutaneous inoculation of 2 × 10^5 LLC cells to the right flank. The criterion of successful preparation was a tumor nodule with a mean diameter of 2-3 mm at the inoculation site. A total of 90 subcutaneous transplanted tumor mice were randomly allocated into control group, tumor cell vaccine group, GM-CSF group, cisplatin group and GM-CSF + cisplatin group (n = 15). On the 8th day after inoculation, control group received normal saline, tumor cell vaccine group received irradiated tumor cell vaccine without express-
Tumor cell vaccine expressing GM-CSF

The formula \[ dB \ (mg/kg) = dA \times RB/RA \times (WA \times WB) \times 1/3. \]
dA (mg/kg) was the dosage of cisplatin for human, RA and RB were the coefficients of animal sizes (RA = 100 for human and RB = 59 for mice), and WA (kg) and WB (kg) were the weight of human and mice, respectively. These mice were then followed up until 60 d after inoculation. The long and short diameter of transplanting tumors were measured with a vernier caliper at the 8th, 11th, 14th, 17th, 20th and 23rd day after inoculation, and the volume of transplanted tumors (V) was evaluated with the formula \[ V = L \times W^2/2 \] [14]. The experimental mice were executed on the 50th d after inoculation, and their tumors were then resected for pathological examinations.

**LLC cell proliferation and apoptosis**

The tumor tissue was fixed in 10% paraformaldehyde (Sigma), dehydrated, and embedded in paraffin. Two pathological sections were prepared for immunohistochemical staining of PCNA and PTEN. All sections were simultaneously checked by three experienced pathologists who were blind to the grouping of mice. For immunohistochemical staining of PCNA and PTEN (× 400), five fields were randomly selected, and the cells dyed and total cells were counted. Immumohistochemical staining of PTEN was used for evaluating LLC cell apoptosis, and PCNA was used for evaluating LLC cell proliferation. The formula for calculating apoptosis index was apoptosis index = (PTEN + cells/total cells) × 100%, and the formula for proliferation index was proliferation index = (PCNA + cells/total cells) × 100%.

**Western blot**

Whole cell extracts (30 μg) of the tumor tissue of subcutaneous transplanted tumor mice were separated for PCNA by 12% SDS-PAGE and for PTEN by 10% SDS-PAGE, and then transferred onto PVDF membranes (Millipore). The membranes were blotted with rabbit antibodies to GAPDH, PTEN (1:500; GeneTex, Inc., San Antonio, Texas, USA) and PCNA (1:2500; GeneTex, Inc., San Antonio, Texas, USA) overnight, and

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**Figure 1.** Construction of recombinant adenovirus Ad5-GM-CSF-EGFP and Morphology of HEK293 cell transfected with Ad5-GM-CSF-EGFP: A: For electropherogram of recombinant adenovirus Ad5-GM-CSF-EGFP, and 1 for positive control, 2 for Ad5-GM-CSF-EGFP, 3 for negative control, and 4 for Marker DL 2000; B: For Cell morphology of HEK293 cells transfected with Ad5-GM-CSF-EGFP, and 1 for normal HEK293 cells (original magnification, × 200) and 2 for HEK293 cells transfected (original magnification, × 200); C: For Green fluorescent protein in Ad5-GM-CSF-EGFP in transfected HEK293 cells (original magnification, × 100).

**Table 1.** Growth inhibitory rates of LLC cells, LLC cells transfected with Ad5-GM-CSF and Ad-control

<table>
<thead>
<tr>
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<th>0 h (%)</th>
<th>24 h (%)</th>
<th>48 h (%)</th>
<th>72 h (%)</th>
<th>96 h (%)</th>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>0.61</td>
<td>0.77</td>
<td>0.80</td>
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<tr>
<td>Ad5-GM-CSF</td>
<td>0</td>
<td>34.75*</td>
<td>51.58*</td>
<td>64.36*</td>
<td>70.94*</td>
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*: P<0.05, vs LLC; #: P<0.05, vs Ad5.
Tumor cell vaccine expressing GM-CSF

followed by HRP-conjugated anti-rabbit IgG antibody (1:10000; GeneTex, Inc., San Antonio, Texas, USA), respectively. The signal was detected with enhanced chemiluminescence detection system (Pierce, Rockford, IL) as described by the manufacturer.

Statistical analysis

Statistical analysis was performed using the SPSS version 19.0 for Windows (SPSS Inc., USA). Data were expressed as mean ± SD and compared with one-way ANOVA following post hoc. The Kaplan-Meier plot for survival was assessed for significance using the log-rank test. Significance was set at \( P < 0.05 \).

Results

Construction of recombinant adenovirus Ad5-GM-CSF-EGFP

Recombinant adenovirus Ad5-GM-CSF-EGFP containing GM-CSF was constructed (Figure 1A), and the result of DNA sequencing showed that the sequence of GM-CSF in Ad5-GM-CSF-EGFP was completely consistent with mouse GM-CSF gene (gi:51100).

Morphology of HEK293 cell transfected with Ad5-GM-CSF-EGFP

HEK293 cells were co-transfected by shuttle plasmid pDC316-CMV-EGFP-GM-CSF and skeleton adenovirus plasmid pBHGlox(delta)EI with 3Cre. Virus plaque and typical CPE appeared on the 7th day after co-transfection (Figure 1B). HEK293 cells were then transfected with virus solution, and green fluorescent protein could be observed after two days (Figure 1C).

Antitumor effect in vitro

GIR was higher for LLC cells transfected with Ad5-GM-CSF than for LLC cells and LLC cells transfected with Ad5 at 24 h, 48 h, 72 h and 96 h, and GIR was not statistically different between LLC cells and LLC cells transfected with Ad5 (Table 1). These results indicated that the
Tumor cell vaccine expressing GM-CSF

Figure 3. Immunohistochemical staining of PTEN and PCNA (original magnification, × 400) in the tumor tissue of subcutaneous transplanted tumor mice. A: For PTEN in control group; B: For PTEN in tumor cell vaccine group; C: For PTEN in cisplatin group; D: For PTEN in GM-CSF group; E: For PTEN in GM-CSF + cisplatin group; and F: For PCNA.

Figure 4. Apoptosis indexes and proliferation indexes of different groups. A: For apoptosis indexes (PTEN + cells/total cells × 100%), *: P<0.05, vs Control group, ※: P<0.05, vs Tumor cell vaccine group; ☆: P<0.05, vs Cisplatin group, ★: P<0.05, vs GM-CSF group; B: For proliferation indexes (PCNA + cells/total cells × 100%), and the difference between different groups was not statistically different (all P>0.05).

tumor cell vaccine modified with GM-CSF could inhibit the growth of LLC cells.

**Antitumor effect in vivo**

GM-CSF + cisplatin group, GM-CSF group, cisplatin group and tumor cell vaccine group had an average tumor volume (Figure 2A) of 1.20 ± 0.23 cm³, 2.24 ± 0.26 cm³, 3.22 ± 0.32 cm³ and 3.37 ± 0.35 cm³ on the 23th day after inoculation, respectively, showing 72.91%, 49.44%, 27.31% and 23.93% tumor inhibition compared with control group (4.43 ± 0.41 cm³), and moreover, GM-CSF + cisplatin group had the smallest volume compared with other groups (Figure 2B). All subcutaneous transplanted tumor mice survived over 25 days and died within
Tumor cell vaccine expressing GM-CSF

60 days after inoculation, GM-CSF + cisplatin group, GM-CSF group, cisplatin group and tumor cell vaccine group had higher survival rates compared with control group, and moreover, GM-CSF + cisplatin group had the highest survival rates compared with other groups (Figure 2C). These results indicated that tumor cell vaccine modified with GM-CSF had an antitumor effect and the effect could be enhanced through combination with cisplatin.

Pathological results

Immumohistochemical staining of PCNA and PTEN were showed in Figure 3. The apoptotic index (PTEN+ cells/total cells × 100%) was higher in GM-CSF + cisplatin group, GM-CSF group, cisplatin group and tumor cell vaccine group than in control group, and moreover, GM-CSF + cisplatin group had the highest apoptosis index compared with other groups (Figure 4A). The proliferation index (PCNA+ cells/total cells × 100%) was not statistically different between different groups (Figure 4B).

Western blot results

Western blotting analysis showed that the expression level of PTEN was higher in GM-CSF + cisplatin group, GM-CSF group, cisplatin group and tumor cell vaccine group than in control group, and moreover, GM-CSF + cisplatin group had the highest apoptosis index compared with other groups (Figure 5). The expression level of PCNA was not statistically different between different groups (Figure 5).

Discussion

GM-CSF is mainly produced by activated T cells and macrophages, which may promote the differentiation and maturation of dendritic cells and up-regulate the expression of costimulatory molecules for stimulating antitumor immune response [15]. In vitro, GM-CSF may promote the proliferation of myeloid progenitor, enhance the phagocytosis of neutrophils, mononuclear macrophages and eosinophils for tumor cells and antibody dependent cell-mediated cytotoxicity (ADCC), and promote the infiltration of CD4 T cells, CD8 T cells and NK cells at the tumor site [16, 17].

Lung cancer cell vaccine modified with GM-CSF may induce specific immune responses and enhance the killing activity of splenocytes for LLC cells in mice [18]. Moreover, dendritic cell vaccine modified with GM-CSF may also induce a specific killing effect for tumor cells in vitro [19]. In our study, LLC cells transfected with Ad5-GM-CSF had a dramatically decreased proliferation compared with LLC cells and LLC cells transfected with rAd5 in vitro, and LLC cell vaccine modified with GM-CSF could significantly inhibit tumor growth and prolong survival of subcutaneous transplanted tumor mice compared with control group and LLC cell vaccine group, which indicated that this tumor cell vaccine modified with GM-CSF had a higher antitumor activity.
Tumor cell vaccine expressing GM-CSF

Recently, the combination of GM-CSF gene with other genes is frequently used to modify tumor vaccine with the aim of enhancing the antitumor activity of immunotherapy. LL/2 tumor cell vaccine modified with a co-expressing mouse GM-CSF and IL-18 plasmid may significantly inhibit tumor growth and prolong survival of the mice bearing LL/2 tumor whether adoptive or prophylactic immunotherapy in vivo [8]. A double recombinant vaccinia virus (VV-GM-CSF-Lact) may induce the death of cancer cells more efficiently than recombinant VACV coding only GM-CSF, showing an enhanced antitumor activity [20]. A DNA vaccine expressing MUC1 and VEGFR2 with GM-CSF as an adjuvant has an enhanced synergistic effect of anti-Lewis lung carcinoma, and the mice vaccinated with this DNA vaccine may significantly inhibit the growth of MUC1-expressing tumors and prolong the survival of the mice [21]. In addition, a phase I/IIa trial on E39 (GALE 301) + GM-CSF, an HLA-A2-restricted, FBP-derived peptide vaccine shows that the combination GM-CSF with E39 may induce a dose-dependent, strong immune response and is well-tolerated in vivo [22]. In addition to as the adjuvant of tumor vaccine, the combination of lung cancer cell vaccine expressing GM-CSF with pacilitaxel chemotherapy may enhance the effect of immunotherapy for mice bearing transplanted Lewis lung carcinoma [23]. In this study, the antitumor effect of combined LLC cell vaccine expressing GM-CSF with cisplatin was analyzed. Our results showed that the combination of the tumor cell vaccine expressing GM-CSF with cisplatin had an enhanced inhibition for tumor growth and prolonged survival for subcutaneous transplanted tumor mice compared with other groups, which showed that GM-CSF might have a synergy in combination with cisplatin. Furthermore, the potential mechanism for the antitumor activity of GM-CSF was that it could promote the apoptosis of tumor cells, which was also confirmed by the western blot results of PTEN and PCNA.

In summary, we successfully prepared Lewis lung cancer cell vaccine modified with GM-CSF and then confirmed its higher antitumor activity compared with Lewis lung cancer cell vaccine. Furthermore, we confirmed that combined therapy of LLC cell vaccine expressing GM-CSF with cisplatin had an enhanced antitumor effect for subcutaneous transplanted tumor mice.

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Disclosure of conflict of interest

None.

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References


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