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Delivery of human NKG2D-IL-15 fusion gene by chitosan nanoparticles to enhance antitumor immunity

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ABSTRACT

Nanoparticles are becoming promising carriers for gene delivery because of their high capacity in gene loading and low cell cytotoxicity. In this study, a chitosan-based nanoparticle encapsulated within a recombinant pcDNA3.1-dsNKG2D-IL-15 plasmid was generated. The fused dsNKG2D-IL-15 gene fragment consisted of double extracellular domains of NKG2D with IL-15 gene at downstream. The average diameter of the gene nanoparticles ranged from 200 nm to 400 nm, with mean zeta potential value of 53.8 ± 6.56 mV. The nanoparticles which were loaded with the dsNKG2D-IL-15 gene were uptaken by tumor cells with low cytotoxicity. Tumor cells pre-transfected by gene nanopartilces stimulated NK and T cells in vitro. Intramuscular injection of gene nanoparticles suppressed tumor growth and prolonged survival of tumor-bearing mice through activation of NK and $CD8^+$ T cells. Thus, chitosan-based nanoparticle delivery of dsNKG2D-IL-15 gene vaccine can be potentially used for tumor therapy.

1. Introduction

Exogenous genes can be delivered into patients to treat genetic diseases or suppress tumor growth [1,2]. Nanoscale-sized particles have been used in diagnosis and treatment of many diseases. The polymeric nanoparticle, nanohydrogel, shows several advantages. Nanohydrogels are physically or chemically cross-linked hydrophilic polymer networks with huge loading capacity of water-soluble compounds. These particles show high biostability in physiological media, with distinct responsiveness to environmental factors. They are safe and can be degenerated without toxicity in hosts [3,4]. Thus, they are versatile tools for the delivery of peptides, carbohydrates, and oligonucleotides.

NKG2D, which is widely expressed by NK, $CD8^+$T, γδ T, and NKT cells, functions as an important activating receptor. NKG2D ligands include retinoic acid early induced transcript-1, H60, and murine ULBP-like transcript 1 in mouse, as well as MICA/B and ULBPs (UL-16 binding proteins) in humans [5,6]. These ligands are preferentially expressed in stress-induced cells or tumor cells and absent in most normal tissues, thereby serving as potential targets in tumor immunotherapy [7,8]. Moreover, serum soluble MICA/B and ULBP concentrations increase in late-stage cancer patients. These soluble ligands suppress antitumor immunity through competitive binding with NKG2D and downregulation of NKG2D expression in lymphocytes [9,10].

IL-15 is an essential cytokine for activation and survival of NK and $CD8^+$T cells [11]. Exogenous IL-15 has been evaluated for tumor therapy in mice and clinical studies [12,13]. We previously generated two recombinant fusion proteins, namely, human dsNKG2D–IL-15 (hdsNKG2D-IL-15) and mouse dsNKG2D-IL-15 (mdsNKG2D-IL-15), in which two identical NKG2D extracellular domains were fused to IL-15 [14]. DsNKG2D-IL-15 binds to NKG2D ligand-positive tumor cells through double NKG2D extracellular domain and activates NK or $CD8^+$ T cells via the IL-15 moiety,

Abbreviations: MICA, major histocompatibility complex class I chain-related protein A; DD, degree of deacetylation; PBS, phosphate buffer saline; NP, nanoparticle; DLS, dynamic light scattering; ELISA, enzyme-linked immunosorbent assay.

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thereby retarding tumor growth and enhancing the survival of tumor-bearing mice.

Chitosan is one of the most promising polymers for drug delivery because of its polycationic, biocompatible, and biodegradable nature [15,16]. In the present study, we aimed to generate chitosan-based nanoparticles to deliver the dsNKG2D-IL-15 fusion gene, which was inserted into a eukaryotic expression vector. Physical and chemical characterizations, biosafety, and transfection efficiency of nanoparticles were evaluated in vitro, and the antitumor activity of nanoparticles in tumor-bearing mice was observed.

2. Materials and methods

2.1. Plasmids, cell lines, and reagents

The pcDNA3.1 (−) plasmid was obtained from Clontech (Mountain View, CA, USA). The recombinant pQE3.1-dsNKG2D-IL-15 plasmid was previously generated [14]. *Taq* DNA polymerase, restriction endonucleases, T4 DNA ligase, PCR product purification, and DNA recovery kits were all obtained from Takara Bio (Dalian, China). Lipofectamine™ 2000 was obtained from Invitrogen (Grand Island, NY, USA). EndoFree Plasmid Maxi Kit was purchased from Qiagen (Düsseldorf, Germany). Chitosan, (molecular weight, 20 kDa) with deacetylation degree (DD) of 75%–85%, was obtained from Yuhuan County Marine Chemical Company (Yuhuan, Zhejiang, China). IL-15 antibody (H-114) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against CD8 (53.67), NK1.1 (PK136), CD69 (LG.3A10), NKG2D (CX5), and CD44 (IM7) were purchased from Biolegend (San Diego, CA, USA). Secondary antibodies conjugated to a fluorophore were obtained from Invitrogen (Grand Island, NY, USA). B16BL6, RAW264.7, and CT-26 cell lines were obtained from ATCC. MICA was expressed ectopically on B16BL6 cells, as previously described [17].

2.2. Preparation of chitosan-plasmid nanoparticles

Human dsNKG2D-IL-15 fusion gene fragment was cut from pQE31-dsNKG2D-IL-15 plasmid and inserted into pcDNA3.1 (−) in accordance with usual practice. DNA fragment of signal peptides from β2-microglobulin was also added at the upstream of NKG2D gene. Large amounts of pcDNA3.1-dsNKG2D-IL-15 plasmid were extracted and purified using plasmid extraction columns. Chitosan was chemically modified with N-(2-hydroxyl) propyl-3-trimethyl ammonium [18]. Chitosan and plasmids were separately dissolved in ultrapure water to obtain 1 mg/mL solutions. The plasmid solution was added dropwise into chitosan solutions and mixed at weight ratios of 1:1, 2:1, or 1:2. Mixed liquids were shaken at 300 rpm for 30 min. The mixture was centrifuged to form sediments. Sediments were collected and dissolved with phosphate-buffered saline for future use.

2.3. Physicochemical characterization of chitosan-plasmid nanoparticles

The amount of plasmid present in the nanoparticles was determined by the difference of total amount used and the amount present on the supernatant, which was detected using an ultraviolet spectrophotometer. Free plasmids in the supernatants were verified via agarose gel electrophoresis. Particle size and zeta potential of the chitosan/DNA complexes were detected by dynamic light scattering and zeta potential measurements in aqueous solutions on a Zetasizer Nano ZS 90 (Malvern, UK). The sizes of nanoparticles were confirmed using an electron microscope.

2.4. Transfection with chitosan-plasmid nanoparticles

B16BL6 (a murine melanoma cell line) or RAW264.7 (a murine macrophage cell line) cells were previously plated onto a 24-well plate with 10^5 cells. At the time of transfection, the culture medium of each well was replaced with serum-free DMEM medium. Chitosan-plasmid nanoparticles were added into plates at final dilutions of 10% or 20% by DMEM. No-load chitosan nanoparticle was used as negative control. After 6 h, the serum-free culture medium was removed, and a complete medium was added. Following 48 h cultures, cell supernatants were collected for assessment of dsNKG2D-IL-15 secretion by B16BL6 or RAW264.7 cells. ELISA kit from R&D Systems (Boston, MA, USA) was used to determine IL-15 concentration in accordance with the manufacturer's protocol. The viability of both cell lines cultured with chitosan-plasmid nanoparticles was assessed with an MTS/PMS kit from Promega (Madison, WI, USA). After adding various doses of no-load chitosan particles (1.25, 2.5, and 5 μg) and chitosan-plasmid particles (1.25, 2.5, and 5 μg) into cell wells for 24 h, the MTS/PMS solution was added to each well. After 4 h, absorption data at 490 nm were detected to evaluate the viable cell numbers.

2.5. Immunofluorescent staining

CT-26 cells (murine colon cancer cell line), which were pre-transfected with pcDNA3.1-dsNKG2D-IL-15 or pcDNA3.1 by liposome, were added into the sections. After fixing with cold acetone, the sections were blocked with donkey serum and stained with IL-15 antibody and fluorescent-labeled goat anti-rabbit IgG secondary antibody. With complete wash, the sections were covered with 30 μL of mounting medium with DAPI (Vector Labs). Fluorescence was detected using Eclipse E600 (Nikon).

2.6. Mouse tumor models

B16BL6-MICA cells ($n = 6$; 2×10^6 cells) were implanted subcutaneously into the rear of C57BL/6 mice. After 7 d, the mice were treated daily with chitosan-dsNKG2D-IL-15 gene nanoparticles (100 μg) via intratumoral or intramuscular injection. Recombinant human dsNKG2D-IL-15 protein (60 μg) was used as positive control. Tumor growth was measured with digital calipers, and tumor volume was calculated as $V = (\text{width})^2 \times \text{length}/2$. All the mice were killed on day 21, and their spleens were removed. Splenocytes were isolated, and CD69 on NK and NKG2D on $CD8^+$T cells were detected via flow cytometry. To evaluate the tumor-bearing mice survival, mouse viability was recorded daily. All the experiments were conducted in accordance with protocols approved by the Yangzhou University's Institutional Animal Care and Use Committee.

2.7. Statistical analysis

Differences between groups were analyzed by Student's *t*-test. Values of $P < 0.05$ were considered significant. Kaplan–Meier survival curves were plotted and analyzed with GraphPad Prism software.

3. Results

3.1. Identification of recombinant pcDNA3.1-dsNKG2D-IL-15 plasmid

Recombinant eukaryotic expression vector, pcDNA3.1-dsNKG2D-IL-15, was identified by digestion of double restrictive enzymes. With digestions by BamH I/Kpn I, BamH I/Hind III, or Hind

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