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Anti-tumor effects in mice induced by survivin-targeted siRNA delivered through polysaccharide nanoparticles

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ABSTRACT

Recently, survivin has been attracting great attention because it plays an important role in inhibiting the apoptosis process of tumor cells. Down-regulating the expression of survivin gene by small interfering RNA (siRNA) offers a promising method for anti-tumor therapy. However, lack of appropriate siRNA delivery vector has significantly hindered the successful application of survivin-targeted siRNA in anti-tumor therapy. The purpose of this study was to use polysaccharide vector TAT-g-CS we synthesized to deliver functional siRNA and evaluate its in vivo anti-tumor activity. TAT-g-CS vector was firstly synthesized and well structurally characterized. MTT assay showed that TAT-g-CS vector exhibited good biocompatibility. TAT-g-CS complexed with siRNA offering nanoparticles with an average particle size of 212.2 nm and a polydispersity index of 0.121, and the zeta potential of the nanoparticles was +18.58 mV. Results from reporter gene assay suggested that luciferase-targeted siRNA when delivered by TAT-g-CS could down-regulate the expression of luciferase gene with 75.3% reduction. Most importantly, we use siRNA^{Sur} targeting survivin gene to assess the in vitro and in vivo delivery capacity of TAT-g-CS and its anti-tumor effects. Our results demonstrated that TAT-g-CS/siRNA^{Sur} nanoparticles not only strongly inhibited the in vitro proliferation of 4T1-Luc tumor cells via inducing cell apoptosis, but also effectively inhibited the in vivo growth and metastasis of malignant breast tumor, which suggested that TAT-g-CS/siRNA nanoparticle was a highly efficient non-viral system for siRNA delivery, especially for anti-tumor therapy based on siRNA therapeutics.

1. Introduction

Malignant tumors, namely cancers, are fatal diseases which severally threaten the health of human beings. It was statistically reported that about 8 million people died from various kinds of cancers every year in the world, including breast cancer, lung cancer, live cancer, brain cancer and so on [1,2]. Therefore, tremendous efforts have been paid to explore effective therapeutic modalities for anti-tumor therapy. Recently, survivin has been attracting great attention because it was found that survivin is over-expressed in the majority of malignant tumors [3], but expressed quite lowly in normal tissues [4]. It was demonstrated that survivin is a member of the inhibitor of apoptosis (IAP) family [5], and the expression of survivin was up-regulated in tumor cells [6], As a whole, survivin plays a very important role in inhibiting the apoptosis process of tumor cells [7]. Therefore, survivin has been confirmed as a promising pharmacological target in cancer treatment. Currently, down-regulating the expression of survivin gene becomes an effective therapeutic modality for cancers [8].

Small interfering RNA (siRNA) is one type of double-stranded RNA with a length of 20–25 base pairs [9]. By inducing RNA interference response, siRNA can specifically down-regulate the expression of target genes [10], which provides a promising therapeutic method for serious diseases, especially various cancers [11].

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Recently, survivin-targeted siRNA have been designed and employed to silence the survivin gene to inhibit the tumor growth [12]. Naked siRNA when used alone shows very poor intracellular uptake and is easily degraded by nuclease [13], so vectors are needed to protect and deliver siRNA into cells to exert its therapeutic effects [14]. Up to now, the lack of appropriate delivery vector has become a major hurdle in the development of siRNA therapy for severe diseases including cancers [15]. Although transfection is highly efficient, viral vectors are strictly limited due to its immunogenicity, potential pathogenicity and laborious preparation [16]. In contrast, non-viral vectors draw high attention because of their advantages such as easy preparation, good biocompatibility, non-pathogenicity and non-immunogenicity [17].

It is well known that chitosan has been used as non-viral vector to deliver plasmid DNA and siRNA [18]. At the same time, a lot of derivatives of chitosan also have been explored to deliver siRNA [19]. Recently, it has been reported that TAT peptide, an excellent cell-penetrating peptide, was conjugated onto the surfaces of chitosan nanoparticles for siRNA delivery [20]. However, in this report, the delivery potential of TAT surface-modified chitosan nanoparticles has not been investigated. Moreover, this kind of surface modification showed a few disadvantages. For example, the content of conjugated TAT peptides cannot be determined, and there is also a difficulty in purification of TAT modified nanoparticles. In the present study, we directly grafted the TAT peptides at the site of the primary amino groups of chitosan (CS) molecules to synthesize the copolymer TAT-g-CS as non-viral vector for siRNA delivery. The structure composition and cytotoxicity of copolymer TAT-g-CS were well characterized. Importantly, we used TAT-g-CS to load survivin-targeted siRNA to prepare nanoparticles for anti-tumor therapy, and the anti-tumor effects of such nanoparticles in vitro and in vivo were investigated in detail.

2. Materials and methods

2.1. Materials

Chitosan (Mw 11.3 kDa) was purchased from FMC Biopolymer. Carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were obtained from Aladdin company. The 3-(4,5)-dimethylthiazol(-z-y1)-3,5-di-phenytetrazoliumromide (MTT) was obtained from AMRESCO company. Trypsin, fetal bovine serum, DMEM and RPMI-1640 mediums and were provided by Hyclone company. The Cell Counting Kit-8 was purchased from Dojindo company. Propidium Iodide (PI) was from Beyotime Institute of Biotechnology (Haimen, China). Luciferase Reporter Assay System kit was purchased from Promega. Annexin V–FITC apoptosis detection kit was provided by KeyGEN biosciences company. Other chemicals and reagents were of analytical grade.

2.2. Synthesis of peptide and siRNA

TAT peptide with the amino acid sequence of GCGGGYGRKKRRQRRR was synthesized by GL Biochem Ltd (Shanghai, China). The duplexed siRNA^{Luc} and siRNA^{Sur} with the following sequences were synthesized by Dharmacon company. The sequences of siRNA^{Luc} and siRNA^{Sur} were designed to target the luciferase and survivin genes, respectively.

siRNA^{Luc}: 5′-CUUACGCUGAGUACUUCGATT-3′.

siRNA^{Sur}: 5′-GAACAUCAUCAUCCAGGAC-3′.

2.3. Synthesis and structural characterization of TAT-g-CS

The synthesis route of copolymer TAT-g-CS was showed in Fig. 1. TAT-g-CS was synthesized through a coupling reaction between primary amine groups in CS molecules and carboxyl groups of TAT peptide using EDC and NHS as coupling reagents. Briefly, 40 mg CS, 45 mg EDC and 40 mg NHS were firstly dissolved in 10 mL of acetic acid/sodium acetate (HAc/NaAc) buffer solution (pH5.5), and 76.8 mg of TAT peptide was added subsequently. The mixture solution was stirred at room temperature for 24 h for the coupling reaction to synthesize TAT-g-CS. Then, the reaction solution was dialyzed against the deionized water for 48 h. Finally, the reactive product of TAT-g-CS was obtained by lyophilizing the dialyzed reaction solution. The structure of synthetic copolymers was characterized with Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR). For FTIR measurement, samples were firstly compressed into KBr tablets, and then analyzed with a Nicolet 5700 FTIR spectrometer (Thermo) in the range of 600–4000 cm^{-1} with a resolution of 4 cm^{-1}. The ^1H NMR analysis was conducted by accumulation of 1000 scans at a relaxation time of 2 s on a Bruker Mercury-500 MHz spectrometer (Varian) at room temperature using D_2O as the solvent.

2.4. Cell line and cell culture

Human breast adenocarcinoma cell line MCF-7-Luc and mouse mammary tumor cell line 4T1-Luc were provided by the Department of Pathology in Institute of Medicinal Biotechnology in Peking Union Medical College. These cell lines were labeled with luciferase reporter gene, and can stably express the firefly luciferase. MCF-7-Luc cell line was cultured with DMEM medium containing 10% fetal bovine serum at 37 °C in humidified atmosphere containing 5% CO_2. Similarly, 4T1-Luc cell line was cultured in RPMI-1640 medium with 10% fetal bovine serum at 37 °C and 5% CO_2. The cell sub-culture was carried out once cells grew to about 70–80% confluence. Cells in logarithmic growth phase were used to conduct all cell experiments in this study.

2.5. Cytotoxicity of TAT-g-CS copolymer

The cytotoxicity of TAT-g-CS copolymer was evaluated through MTT assays in MCF-7-Luc and 4T1-Luc cells, respectively. Firstly, cells were seeded into a 96-well plate at a density of 5×10^3/well. Blanks were prepared by adding culture medium alone. Cells were cultured overnight for attachment. Subsequently, culture medium in each well was replaced with fresh medium containing TAT-g-CS copolymer in series of concentrations from 5 to 100 μg/mL. Cells in wells without addition of TAT-g-CS copolymer sample were used as control group. Six replicates were included in each group. After another 12-h culture, 20 μL of sterile MTT solution (5 mg/mL) was added into each well to incubate at 37 °C for 4 h. After the removal of the unreacted MTT dye by aspiration, 150 μL of DMSO was added into each well to dissolve the produced formazan crystals, and the plate was gently shaken for 10 min. Finally, the optical density (OD) was measured at 490 nm using the SpectraMax 190 Absorbance Microplate Reader (Molecular Devices, USA). The cell viability was calculated according to the below formula.

$$\text{Cell viability}(\%) = \left[\left(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}\right) \Big/ \left(\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}\right)\right] \times 100\%$$

2.6. Preparation and characterization of TAT-g-CS/siRNA nanoparticles

The TAT-g-CS/siRNA nanoparticles were prepared through the complex coacervation method. Firstly, TAT-g-CS copolymers were dissolved in HAc/NaAc buffer (pH5.5) at a concentration of 200 μg/mL, and then the TAT-g-CS solution was filtered for sterilization using 0.22 μm membranes. The siRNA stock solution was diluted with RNase-free water to a concentration of 1 pmol/μL. Then, TAT-g-CS and siRNA solutions were preheated in a water bath at 55 °C for 20 min, respectively. Subsequently, the equal volume of TAT-g-CS and siRNA solutions were mixed and vortexed immediately for 45 s to generate the TAT-g-CS/siRNA nanoparticles. Particle size and zeta potential of TAT-g-CS/siRNA nanoparticles were determined through dynamic light scattering (DLS) and electrophoretic light scattering (ELS), respectively. The nanoparticles suspensions were diluted with double distilled water to measure the particle size and zeta potential using a Nicomp380/ZLS analyzer (Particle Sizing Systems, USA). The morphology of nanoparticles was characterized by the transmission electron microscopy. Briefly, 5 μL of nanoparticles suspension was dropped onto a copper grid coated with carbon membrane, and then the copper grid was air-dried. Subsequently, the morphology of nanoparticles was observed using a transmission electron microscope (Hitachi H-7650, Japan) with an acceleration voltage of 200 kV.

2.7. Gel retardation assay

The gel retardation assay was carried out by 4% agarose gel electrophoresis. According to the above preparation method, various weight of TAT-g-CS was respectively used to complex 0.9 μg of siRNA at different weight ratios ranging from 30 to 250. The generated nanoparticle suspensions were loaded onto 4% agarose gel and subjected to electrophoresis in TAE buffer at a constant voltage of 55 V for 120 min. Then, the agarose gel was stained with the 0.5 μg/ml ethidium bromide solution for 60 min. Finally, the siRNA bands were visualized at 365 nm using a UV gel image system (SIM135A, SIMON).

2.8. Cellular uptake assay by flow cytometry

The cellular uptake efficiency of TAT-g-CS/siRNA nanoparticles was assayed by flow cytometry. MCF-7-Luc cells were seeded into a 6-well plate and cultured for attachment. Then, the nanoparticles loading FAM-labeled siRNA^{Luc} were added into wells to incubate at 37 °C for 0.5 h. After collecting cells in wells by trypsin digestion and centrifugation, cells were re-suspended in 0.2 mL PBS buffer to be analyzed using a flow cytometer (EPICS XL, Beckman Coulter).