Biomaterials 31 (2010) 4426–4433

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Reversion of multidrug resistance by tumor targeted delivery of antisense oligodeoxynucleotides in hydroxypropyl-chitosan nanoparticles

Jiaqi Wang [1], Xinyi Tao [1], Yufei Zhang, Dongzhi Wei, Yuhong Ren*

State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China, 200237

ARTICLE INFO

Article history:
Received 21 December 2009
Accepted 3 February 2010
Available online 25 February 2010

Keywords:
Hydroxypropyl-chitosan nanoparticles
Tumor-targeted
Antisense oligodeoxynucleotides
Multidrug resistance

ABSTRACT

Chitosan and its derivatives have shown great potential as non-viral vectors for gene delivery therapy. Folic acid receptor (FR) is an important anti-cancer therapy target that is applicable to many cancer types. In this study, we developed an efficient and targeted delivery of antisense oligodeoxynucleotides asODNs, using folic acid (FA) conjugated hydroxypropyl-chitosan (HPCS). These nanoparticles were designed to reduce production of P-gp, in order to overcome tumor drug resistance. Nanoparticles prepared were found to be 181 nm in diameter. Spectrofluorimetry was utilized to evaluate the effect of charge ratio of the nanoparticles on loading efficiency. In PBS buffer, 40% of asODNs were released from the nanoparticles at first 24 h. However, just another 15% was released between 24 and 48 h. The antitumor effect of the nanoparticles was evaluated in KB-A-1 cells implanted in Balb/c-nu/nu mice. They inhibited the growth of tumor by 35% compared to the bare asODNs. The FA–HPCS–asODNs nanoparticles demonstrated significantly inhibition of the multi drug resistance (MDR) 1 gene levels and P-gp levels *in vitro* and *in vivo*, respectively, related with bare asODNs and HPCS–asODNs ones. During *in vivo* studies, FA–HPCS–asODNs nanoparticles were also found to bind specifically and efficiently to FR high-expressing cancer cells. These results suggested that the use of targeted, antisense agent nanoparticles would be potential approach to overcome tumor drug resistance.

1. Introduction

Multidrug resistance (MDR) limits the success of many anti-cancer drugs in cancer chemotherapy. A variety of mechanisms at the cellular level result in drug resistance. Overexpression of P-glycoprotein (P-gp), a drug efflux transporter encoded by the mdr-1 gene, is a key factor contributing to the development of MDR [1]. In many cancer types, it is estimated that, nearly 40–50% of the patients have P-gp overexpression in the malignant tissue [2].

Antisense technology, one aspect of gene therapy, has emerged as a potential therapy for cancer [3]. The ambitious application of antisense oligodeoxynucleotides (asODNs) treatment depends upon efficient knockdown of targeted transcripts. However, asODNs has not shown significant efficacy due to several problems such as its instability in body fluids, non-specificity to the target cells, degradation by enzymes and its low transfection efficiency [4].

Currently, gene therapy can be accomplished by using viral and non-viral vectors. Comparing the limitations of viral vectors regarding safety concerns such as immunogenicity [5], clinical application of gene therapies requires the evaluation and development of alternative non-invasive delivery systems. Among non-viral vectors, chitosan and its derivatives have been developed as a good gene carrier candidate [6], since they are low toxic biodegradable polymers with high cationic potential [7,8]. However, the transfection of the chitosan–DNA nanoparticles is still very low. The transfection efficiency may depend on several factors such as the degree of deacetylation (DDA) [9], molecular weight (M_w) [10] of the chitosan, pH [11], charge ratio of chitosan to DNA [12]. DNA binding efficacy decreased with decreasing DDA. Mao et al. [13] reported that when chitosan/DNA complexes were prepared with highly deacetylated chitosan (above 80%), showed high transfection efficiency. Huang et al. [14] reported that low deacetylated chitosan/DNA complexes were less efficient at retaining the DNA upon dilution, and consequentially, less capable of protecting the condensed DNA from degradation by DNase and serum components.

The use of low molecular weight chitosan resulted in low transfection efficiencies. In order to improve the transfection efficiency, chemical modification of chitosan as gene carriers are also proposed. Park et al. [15] coupled dextran as a hydrophilic group into galactosylated chitosan to enhance its stability and protect the complexes from self-aggregation and precipitation. Lin et al. [16] prepared *N*-dodecylated chitosan complexed with DNA as hydrophobic modification to increase the stability of DNA. Wong et al.

* Corresponding author. Tel.: +86 21 64252981; fax: +86 21 64250068.
E-mail address: yhren@ecust.edu.cn (Y. Ren).
[1] These authors contributed equally to this work.

0142-9612/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2010.02.007

[17] demonstrated that PEI-graft-chitosan/DNA complexes have improved the transfection efficiency in HeLa 293I and HepG2 cell lines due to the presence of water-soluble chitosan. In addition, chitosan can be interacted with specific ligands to enhance the transfection efficiency via binding to the receptors of the cells. Lin et al. [18] developed chitosan nanoparticles modified with glycyrrhizin (CS-NPs-GL) to develop hepatocyte-targeted delivery vehicles,

Folic acid receptors (FR) are over-expressed on many human cancer cell surfaces [19]. Alberto et al. [20] studied several tumor models overexpressing FR, including mouse M109 carcinoma, KB human epidermal carcinoma cell line [21], and mouse J6456 lymphoma. Robert et al. [22] found that uptake of folate–PEG–liposomal DOX by KB cells was 45-fold higher than that of non-targeted liposomal DOX. Lee et al. [23] found that FA-conjugated chitosan showed higher transfection activity than unmodified chitosan.

The aim of this study was to investigate the efficiency of FA-conjugated hydroxypropyl-chitosan nanoparticles asODNs targeting the drug-resistant tumor cells. The resulting nanoparticles were characterized regarding their size, loading efficiency and *in vitro* release. In addition, our studies showed that nanoparticles effectively overcome the tumor drug resistant through the analysis of mdr1 gene and P-gp expression *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Chitosan (Molecular Weight: 201 kDa; Degree of deacetylation: 92%) was purchased from Golden-Shell Biochemical Co., Ltd (Zhejiang, China). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was obtained from Sigma–Aldrich (Shanghai, China). Doxorubicin was obtained from Hisun Pharmaceutical Co., Ltd (Zhejiang, China). 5′-TCCTCCATTGCGGTCCCT-3′ was synthesized by SBSgene (Shanghai, China). Antibodies used in this study were: rabbit anti-human P-glycoprotein (P-gp) and rabbit anti-human b-actin (Cell Signaling) and HRP conjugated goat anti-rabbit secondary antibody was purchased from Univ-Biology Company (Shanghai, China). All other reagents were of analytical grade.

2.1.2. Cell lines

KB-A-1: human carcinoma DOX resistant cells, was a kind gift from Prof. Ira Pastan and Prof. Micheal M. Gottesman (National Institutes of Health, USA). This was cultured in RPMI 1640 medium supplemented with 15% calf serum, 100 U penicillin and 100 μg streptomycin. Cells were grown in a 5–95% CO_2–O_2 atmosphere at 37 °C. All cell culture reagents were obtained from Sigma–Aldrich (Shanghai, China).

2.1.3. Animals and tumors

Balb/c-nu/nu mice were purchased from Slac Laboratory Animal Co. (Shanghai, China). KB-A-1 cells (1×10^6/50 μL in PBS) were subcutaneously injected into one flank of 4–5 week-old mice. The tumors were created for 20 days. The tumor size was measured on alternate days with a vernier calliper and tumor volumes were calculated according to the formula $V = a \times b \times b/2$, where a is the largest and b is the smallest tumor diameter.

2.2. Methods

2.2.1. Conjugation of FA–chitosan (FA–CS)

FA–chitosan was prepared accorded to a previously reported method [5]. Briefly, FA was treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) in anhydrous DMSO and stirred at room temperature until dissolved (2.5 h). 1% (w/v) solution of chitosan (201 kDa) in acetate buffer (0.1 M, pH4.7) was added to FA. The resulting mixture was stirred at room temperature in the dark for 16 h. Then, the solution was brought to pH 9.0 by drop wise addition of 1 M NaOH. The polymer was dialyzed first against phosphate buffer pH 7.4 for 3 days and then against water for 3 days. The final conjugate was isolated by lyophilization and characterized using infrared spectroscopy.

2.2.2. Chemical modification of FA–CS

FA-CS was mixed with isopropanol in 1 M NaOH and stirred for 1 h at room temperature [24]. 5% (w/v) propylene oxide was dropped to the solution and reacted for 5 h at 45 °C. After cooling to room temperature, the mixture was brought to pH 7.0 by drop wise addition of 5 M HCl. The product was precipitated in large amounts of acetone. The polymer was washed with 95% ethanol for 3 times, then anhydrous ethanol for 3 times. The hydroxypropyl-chitosan (HPCS) was dehydrated at 50 °C under vacuum and characterized by infrared spectroscopy.

2.2.3. Preparation for FA–HPCS–asODNs nanoparticles

FA–chitosan–asODNs nanoparticles were prepared as previously reported [1]. FA–chitosan was dissolved in 0.1 M acetate buffer (pH 5.5) and passed through a 0.22 mm filter. AsODNs (330 μg) were diluted in 25 mM sodium sulfate solution. Separately, samples of FA–chitosan and asODNs with N/P ratios between 6 and 10 were diluted to final volume of 50 μL. After incubation at 55 °C for 30 min, the FA–chitosan solution was quickly mixed with the asODNs solution followed by 1 min of vortex. The resulting nanoparticle solution was stored at 4 °C for use.

2.2.4. Particle size characterization

The nanoparticles were suspended in distilled water to achieve an appropriate level of scattering. The particle size and distribution of FA–chitosan–asODNs were measured by Photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern, UK). Mean hydrodynamic diameters were calculated based on size distribution and the results were expressed as mean of three runs.

2.2.5. Encapsulation efficiency and in vitro release

AsODNs in the total nanoparticle solutions (At) with different N/P ratios were diluted to 1 mg/mL. AsODNs unloaded in the supernatant (As) were measured by spectrofluorimetry after centrifugation for 30 min at 12,000 rpm and 4 °C (Ultraviolet–Visible Spectrophotometer 2300, China). Encapsulation dfficiency in nanoparticles was defined as $(1 - \mathrm{As/At}) \times 100\%$.

AsODNs release from nanoparticles was assessed in phosphate buffer saline (PBS, 0.1 M, pH 7.2) at 37 °C. Nanoparticle solutions were diluted with PBS so the asODNs concentration reached 0.33 mg/mL. Then, the suspension was shaken in 1.5 mL Eppendorf tubes using an incubator shaker at 100 rpm and 37 °C. After 24 h and 48 h, nanoparticle suspension was centrifuged for 30 min at 12,000 rpm and 4 °C. Release of asODNs from the nanoparticles was determined by spectrofluorimetry.

2.2.6. Cytotoxicity assay in drug-resistant cells

KB-A-1 cells were seeded in 96-well plates at a density of 1×10^4 cells/well/100 μL. Cells were first cultured for 24 h and then treated with drugs containing FA–HPCS–asODNs, FA–HPCS or HPCS–asODNs at various concentrations for other 24 h. Cell viability was determined using MTT assay. The concentration of DOX was 1 μg/mL 100 μL MTT solution (0.5 mg/mL) was added to each well, and incubated for 4 h at 37 °C. Then, the MTT solution was replaced with 100 μL DMSO. The absorbance at 570 nm for each well was measured on an Automated Microplate Reader 550 (Bio-Rad, USA). Viability of the non-treated control cells was defined as 100%. Cell lethality (%) was calculated using the following formula (Eq. (1)):

$$\text{Cell lethality}(\%) = 1 - \left(\left(\mathrm{OD570_{sample}} - \mathrm{OD570_{blank}}\right) \Big/ \left(\mathrm{OD570_{control}} - \mathrm{OD570_{blank}}\right)\right) \times 100 \quad (1)$$

Where "sample" and "control" mean the cells with and without being treated with nanoparticles, respectively. Optical density at 570 nm blank was the absorbance of the blank wells.

2.2.7. In vivo distribution and targeting study

The KB-A-1 xenograft mice were injected via tail vein with HPCS–asODNs or FA–HPCS–asODNs containing 62 nmol of FAM-labeled asODNs. The mice were sacrificed at 4 h post-injection. Major organs including liver, spleen, kidney and tumors were snap frozen in liquid nitrogen and stored at −80 °C for further analysis. The organs and tumors were cut into 8 μm sections on a Leica CM1900 cryostat. FAM fluorescence was visualized with a fluorescence microscope.

To determine the drug accumulation, tissues, organs and tumors were weighted and homogenized in 0.5 mL PBS solution per 100 mg tissues. Chloroform was added to the homogenate, incubated for 10 min and vortexed for 5 min in order to extract the DNA. After centrifugation at 4000 rpm for 10 min, the fluorescent intensity of the supernatant was determined using a spectrofluorometer at 485 nm and 520 nm of excitation and emission wavelength.

2.2.8. Therapeutic experiment

On day 21 after tumor implantation, animals with visible tumors were randomly divided into four groups: (1) normal saline, (2) DOX in solution (2 mg/kg body weight), (3) asODNs in solution (30 mg/kg) + DOX in solution, (4) FA–HPCS–asODNs (30 mg/kg asODNs) + DOX in solution, (5) HPCS–asODNs (30 mg/kg asODNs) + DOX in solution. The mice were treated via intraperitoneal injection. Treatments were administered every two days until day 17. Tumor volumes were recorded on the treatment day. Body weight of each mouse was also measured at selected days to evaluate any side effects. The mice were sacrificed at the end of experiment and the tumor was excised, and stored at −80 °C for further analysis.

2.2.9. Real-time RT-PCR in human cells and in mice

For the *in vitro* study, KB-A-1 cells were seeded into 6-well cell culture plates. After 24 h, the cells were treated with the desired formulations (no treatment,