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Hyaluronic acid modification of RNase A and its intracellular delivery using lipid-like nanoparticles

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ABSTRACT

Developing safe and effective nanosystems to deliver active and therapeutic proteins to targeted cells and organs is an important tool for many biomedical applications. We present here a simple and efficient strategy for this purpose: delivering hyaluronic acid (HA)-modified RNase A (RNase A-HA) in nanocomplex with cationic lipid-like molecules (lipidoids) to cancer cells, resulting in targeted inhibition of cancer proliferation. The chemical conjugation of RNase A with HA both increased the supramolecular interaction with carrier lipidoids, promoting protein encapsulation efficacy, and facilitated cancer cell targeting via interaction with overexpressed CD44. Through confocal laser scanning microscopy and flow cytometry analysis, we demonstrated that protein/lipidoid nanoparticles could facilely enter cells with high CD44 expression, and inhibit cell proliferation in a dose-dependent manner.

1. Introduction

Protein-based therapeutics have attracted tremendous attention during the last few decades in the treatment of various conditions, including cancer, infection, diabetes, inflammation, and degenerative diseases [1,2]. The advantages of using protein-based therapeutics include high specificity and low off-target effects, compared with traditional small-molecule-based therapies [2]. However, a majority of protein pharmaceuticals, such as cytokines, growth factors, and monoclonal antibodies, carry out their biological activity by targeting cell surface ligands or extracellular domains [2]. Because the cell membrane is largely impermeable to proteins, protein therapeutics with an intracellular target are rare. An effective delivery system for delivering protein to its intracellular target will provide expanded therapeutic modalities for protein-based drugs [3–10]. An example of these modalities may be provided by ribonuclease (RNase) A. RNase A cleaves intracellular RNA, inducing cytotoxic effects upon cell uptake [11]. Frog-derived ribonuclease (Onconase) has already been investigated as a promising therapeutic protein for cancer resistant to traditional chemotherapy [12–14]. However, Onconase is not internalized consistently and specifically by cancer cells [14]. The development of a safe, efficient tool for cancer-targeted intracellular delivery of RNase would therefore dramatically potentiate its activity [11]. For example, Raines et al. have conjugated RNase A with pendant phenylboronic acids (PBA) to mediate its delivery into the cytosol of mammalian cells [15,16]. PBA binds with higher affinity to sialic acid on the surface of mammalian cells, leading to enhanced cytosolic delivery of RNase A.

Recently, we developed an effective approach for RNase A delivery using a combination of reversible chemical modification of protein combined with a cationic lipid-based nanoparticle carrier [17,18]. The chemical modification chosen converts positively charged lysine residues in the protein to negatively charged moieties, allowing for more efficient complexation with cationic lipids. Such nanocomplex could efficiently mediate the intracellular delivery of the protein cargos.

In this paper, we conjugated hyaluronic acid to RNase A, and complexed the modified protein with synthetic lipid-based nanoparticles. Afterwards, we demonstrated targeted intracellular delivery of the resulting molecule to CD44 over-expressing cells, as shown in Scheme 1. The HA modification plays two roles: i) The conjugation "cages" the primary amine groups on the lysine residue, increasing the negative charge density of the protein and facilitating its electrostatic complexation with cationic lipid nanoparticles; ii) HA can specifically bind to the CD44 receptor, which is overexpressed on many solid tumor cell surfaces; therefore, HA conjugation of RNase A offers a cancer targeted delivery scheme [19–24]. We selected the synthetic lipid EC16-80 (chemical structure shown in Scheme 1) for intracellular delivery of

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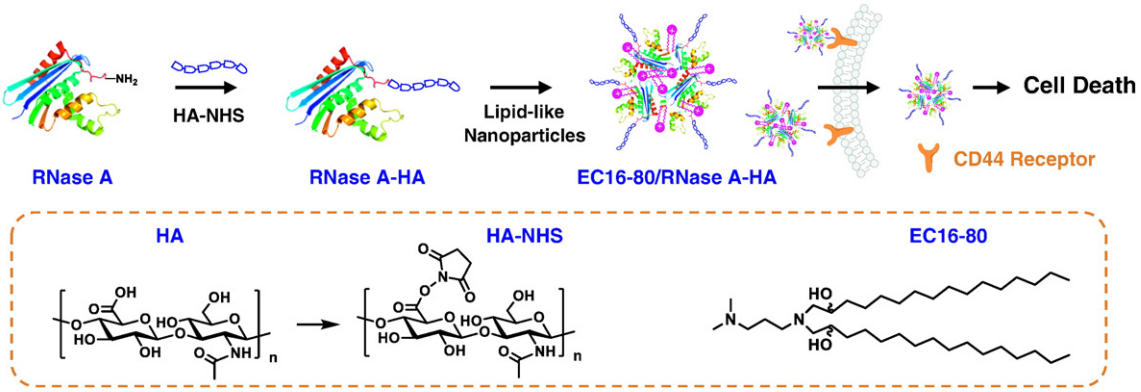

Scheme 1. Schematic illustration of the synthetic route of HA-modified RNase A and CD44-targeted delivery of protein mediated by cationic lipid-like EC16-80 nanoparticles.

RNase A-HA. We have previously delivered several other proteins, including saporin [17], PTEN [25] and chemically modified RNase A [17, 18] using this lipid. Here, we demonstrated that RNase A conjugated with HA was efficiently delivered into CD44-overexpressing A549 cells and significantly inhibited their cell proliferation, indicating that HA-conjugation has great potential for targeted cancer therapy.

2. Materials and methods

2.1. Materials

Bovine pancreatic ribonuclease A (RNase A) was purchased from Sigma-Aldrich. Hyaluronic acid (Research Grade) was purchased from Life Core Biomedical. 1-ethyl-3-(3-diemthylaminopropyl) carbodiimide (EDC) and *N*-hydroxyl succinimide (NHS) were purchased from Sigma-Aldrich. Protein activity of RNase A and RNase A-HA were measured using RNaseAlert® Kit (Integrated DNA Technologies, Inc., IA). Lipidoid EC16-80 was synthesized through the ring-opening reaction of 1,2-epoxyoctadecane and *N*,*N*-dimethyl-1,3-propanediamine according to previous literature [26–28]. BCA protein assay reagents were purchased from Thermo Scientific. Commercially available lipids used for lipidoid/protein nanocomplex formulations (DOPE and C16-PEG2000-ceramide) were purchased from Avanti Polar Lipid, Inc. Monoclonal anti-CD44 antibody was purchased from Sigma-Aldrich (C7923).

2.2. Cell lines and cell culture

MCF-7, human breast adenocarcinoma cells with low CD44 expression, and A549, human lung adenocarcinoma epithelial cells with high CD44 expression [29,30] were originally purchased from ATCC (Manassas, VA, USA). All cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich) and 1% penicillin-streptomycin (Life Technologies) under an atmosphere of 5% CO_2/air at 37 °C.

2.3. Protein modification

RNase A-HA was prepared by reacting RNase A with an excess of hyaluronic acid succinimidyl succinate (HA-NHS). HA-NHS was first prepared by modifying HA with EDC and NHS in a molar ratio of 1/5.73/3.6 (HA/EDC/NHS) in DI water. The pH value of the reaction mixture was maintained at 4.7 by the addition of 0.1 M NaOH/0.1 M HCl; the reaction was allowed to proceed for 24 h at room temperature under continuous stirring. After 24 h, the pH was adjusted to 7.4 with 0.1 M NaOH/0.1 M HCl and RNase A was added into the reaction solution with HA-NHS/RNase A at a molar ratio of 5:1. This reaction was then maintained for 24 h at room temperature and pH 7.5, stirring continuously. The final product RNase A-HA was purified by dialysis (MWCO 15000 Da) against DI water at 4 °C for 3 days, then subjected to bicinchoninic acid assay (BCA), SDS-PAGE, MALDI-TOF, ^{1}H NMR and enzyme activity assays.

2.3.1. BCA test and SDS-PAGE analysis

The protein concentration of RNase A-HA was determined using a Pierce BCA protein assay kit (Thermo Scientific Cat. No. 23227) according to the manufacturer's instructions. SDS-PAGE analysis was also conducted according to the manufacturer's instructions using 4–12% Bis-Tris gel (NuPAGE) and Colloidal Blue Staining Kit (Invitrogen, Cat. No. LC6025).

2.3.2. MALDI-TOF mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was employed to analyze the molecular weights of protein before and after chemical modification from RNase A to RNase A-HA. Samples were prepared by mixing 1 μL of protein solution (1 mg/mL) with 9 μL of matrix solution (saturated sinapinic acid in 50/50 acetonitrile/water with 0.1% trifluoroacetic acid (TFA)).

2.3.3. ^{1}H NMR analysis

The chemical structures of HA, RNase A and RNase A-HA were characterized by ^{1}H NMR analysis, using D_2O as the solvent and a Bruker AVIII 500 MHz NMR spectroscopy.

2.4. Protein activity assay

The protein activities of RNase A and RNase A-HA were measured via the RNaseAlert® Kit according to the manufacturer's instructions [17, 18]. Briefly, 5 μL RNaseAlert® substrate and 10 μL assay buffer (provided by the assay kit) were pre-mixed in a 96-well plate. To the above assay substrate, 85 μL of RNase A or RNase A-HA (2 ng/mL) were added. Then the fluorescence intensity at 520 nm (excited at 490 nm) was monitored within 25 min.

2.5. Lipidoid nanoparticle formulation

Lipidoid/protein (EC16-80/RNase A and EC16-80/RNase A-HA) nanoparticles were formulated by a thin film hydration method, as we have previously described [17,31]. Firstly, lipidoid EC16-80 was mixed with cholesterol and DOPE (Avanti Polar Lipids) at a weight ratio of 16/4/1 (EC16-80/cholesterol/DOPE) and dissolved in chloroform. The chloroform was evaporated under vacuum, and further dried to form a thin film on the bottom of the vial. The film was then hydrated using a mixed solution of ethanol/sodium acetate buffer (200 mM, pH = 5.2, v/v = 9/1). The above solution was then added dropwise to an aqueous solution of C16-PEG2000-ceramide (Avanti Polar Lipids; EC16-80/C16-PEG2000-ceramide = 16/1, w/w). The resulting solution was incubated at 37 °C for 30 min before dialysis (MWCO 3500 Da) against PBS. The as-formulated lipidoid nanoparticle solution was then