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The targeted intracellular delivery of cytochrome C protein to tumors using lipid-apolipoprotein nanoparticles

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ABSTRACT

Intracellular-acting therapeutic proteins offer a promising clinical alternative to extracellular-acting agents, but are limited in efficacy by their low permeability into the cell cytoplasm. We have developed a nanoparticle (NP) composed of lipid (DOTAP/DOPE) and apolipoprotein (APOA-I) to mediate the targeted delivery of intracellular-acting protein drugs to non-small cell lung tumors. NPs were produced with either GFP, a fluorescent model protein, or cytochrome C (cytC), an inducer of apoptosis in cancer cells. GFP and cytC were separately conjugated with a membrane permeable sequence (MPS) peptide and were admixed with DOPE/DOTAP nanoparticle formulations to enable successful protein loading. Protein-loaded NPs were modified with DSPE-PEG-Anisamide to enable specific NP targeting to the tumor site in a xenograft model. The resulting particle was 20–30 nm in size and exhibited a 64–75% loading efficiency. H460 cells treated with the PEGylated MPS-cytC-NPs exhibited massive apoptosis. When MPS-GFP-NPs or MPS-cytC-NPs were intravenously administered in H460 tumor bearing mice, a specific tumor targeting effect with low NP accumulation in the liver was observed. In addition, MPS-cytC-NP treatment provoked a tumor growth retardation effect in H460 xenograft mice. We conclude that our NP enables targeted, efficacious therapeutic protein delivery for the treatment of lung cancer.

1. Introduction

Cancer originates from a deficiency or malfunction in somatic proteins participating in cellular homeostasis. Therapies have been produced that treat cancer by silencing abnormal cell signaling patterns using therapeutic proteins as inhibitors. Compared with gene silencing agents, protein drugs have a rapid onset time and are therefore easily controlled by specific dosing. However, manufactured therapeutic proteins are susceptible to proteolysis, denaturation, and aggregation, limiting their efficacy in the body [1,2].

Nanoparticles have been shown to regulate the release of attached proteins based on the degradation behavior of the NP constituent parts [3–5]. If a therapeutic protein is not highly lipophilic, it is difficult to be encapsulated in this system. However, amphiphilic residues, such as membrane permeable sequences (MPS), can be conjugated to non-lipophilic proteins, including cytC or GFP, enabling these proteins to be associated into the lipid bilayer of NPs [6]. A liposomal nanoparticle matrix offers beneficial protection against enzymatic degradation and antibody neutralization, resulting in prolonged retention of attached protein activity *in vivo* for as long as the proteins remain complexed to their carriers [7]. Recent research has also determined that nanoparticles conjugated with a cell penetrating peptide (CPP) sequence have shown an increased ability to deliver drug cargo to specific cell types after non-specific, systematic treatment [8,9].

Nano-size lipid bilayers have previously been developed to study the function of biomembranes [10,11]. Specifically, nanodiscs, or lipid bilayer nanostructures without aqueous internal spaces, have shown potential for lipophilic drug delivery [12,13]. It has been reported that nanodiscs can serve as easily modifiable matrices with a variable affinity for protein drugs dependent on the relative proportions of lipid or introduced functional group constituents [14].

Previously, we have developed several nano-carrier systems to deliver therapeutic cargoes and have studied the protecting and targeting effect of drug carriers surface-modified with target ligands and PEG [15–17]. In the current study, we have documented a delivery platform that creates a nanoparticle constituted by small, refined lipid bilayers of apolipoprotein and DOTAP/DOPE lipid. Our past results indicate that PEGylation and incorporation of anisamide, a ligand that targets the sigma receptor over-expressed in H460 lung carcinoma, on the surface of the nanoparticle can enable successful evasion of RES-induced, non-specific interactions in the liver [18,19]. In this study, we will detail the therapeutic potential of

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this nanoparticle for delivering MPS-conjugated cytochrome C therapeutic protein into H460 non-small cell lung carcinoma.

2. Materials and methods

2.1. Characterization of MPS-conjugated proteins, MPS-GFP-NPs, and MPS-cytC-NPs

MPS-conjugated protein drugs and NPs were created as described in further detail in the Supplemental Information section. Briefly, MPS peptide (MPS: H-A-A-V-A-L-L-P-A-V-L-L-A-L-L-A-K-OH, 1548 MW from Anaspec, San Jose, CA) was activated with a 1:2 M ratio of protein: EDAC, and the resulting solution was dialyzed. GFP or cytC that had been separately incubated with Alexa-488 succinimidyl esters (Invitrogen) was then reacted with the activated MPS in a 1:2 M ratio for 6 h.

The DOTAP/DOPE/apoA-I nanoparticle was produced using a sodium cholate dialysis method [20,21]. Small unilamellar liposomes were prepared by mixing DOTAP and DOPE (Avanti Polar Lipids, Inc) dissolved in chloroform in a 2:1 M ratio (DOTAP:DOPE), evaporating the solvent, then hydrating the mixture with Tris-HCL buffer and reacting the liposomes with ApoA-I in a DOTAP:DOPE:apoA-I molar ratio of either 50:25:1 or 66:33:1. The solution was incubated overnight until transparent and cholic acids were removed [21,22]. The MPS-GFP or MPS-cytC proteins were then co-incubated with the particles for 1 h to facilitate protein-NP coupling before PEGylation. The MPS-cytC loading efficiency in the NPs was measured using a Sepharose CL-6B column (Amersham Biosciences, Uppsala, Sweden). DSPE-PEG-anisamide was synthesized in our lab as described elsewhere [16,19]. Targeted NPs were prepared by incubating the NP solution (238 μL) with a 15% micellar solution of a 1:1 mixture of DSPE-PEG_{2000} and DSPE-PEG_{2000}-anisamide (10 mg/mL), at 50 °C for 10 min.

2.2. Flow cytometry assay for detection of apoptosis

Estimation of apoptotic NCI-H460 lung cancer cell subpopulations was determined using Annexin V-FITC and PI. Briefly, 1×10^5 cells per sample were washed with PBS, and then were incubated in calcium binding buffer with 3 μL of Annexin V-FITC (0.5 mg/mL) for 20 min in darkness. Cells were washed with PBS, were suspended in 500 μL of calcium binding buffer, then propidium iodide (PI) (5 mg/mL) was added and the samples were immediately analyzed by flow cytometry. All flow cytometric analyses were performed on a BD FACS Canto™ flow cytometer (BD, RUO special order system, CA). A 20 mW blue laser emitting a 488-nm beam served as the excitation source for Annexin V-FITC (FL1) and PI (FL2). Annexin V (green) and PI (red) fluorescent signals were collected with 530 nm and 610 nm band pass filters, respectively, for 10,000 cells per sample.

2.3. Cell imaging by confocal microscopy

NCI-H460 cells were fixed with 2% paraformaldehyde after a 6 h exposure to Alexa488-conjugated MPS-cytC-NPs and a subsequent 1 h incubation with MitoRed (red). Nuclei were counterstained with DAPI (Sigma Aldrich, St. Louis, MO). Confocal images were acquired with an SP2 Laser Scanning Confocal Microscope (Leica, Bannockburn, IL).

2.4. Tissue distribution and tumor uptake study

Female nude mice, 5–6 weeks old, were purchased from NCI. H460 xenograft tumor bearing mice (size 40–50 mm^2) were produced on the 6th day after injection of 5×10^6 NCI-H460 cells on the rear side of the back of mice. In a preliminary study, mice exhibiting tumors ~0.5 cm × 0.5 cm in size were i.v. injected with 160 μg/kg of the MPS-GFP or GFP nanoparticles in different formulations. In a separate study, mice exhibiting the same size tumors were i.v. injected with MPS-cytC or cytC-NPs in different formulations (160 μg/kg). After 4 h, mice were sacrificed and tissues were collected and imaged by the IVIS™ Imaging System (Xenogen Imaging Technologies, Alameda, CA). Samples were fixed in a 10% formaldehyde solution for 2 h and were then washed with PBS. Each treatment was replicated in three mice to ensure statistical significance. The fluorescence intensity of all organs was calculated from the images using manual ROI in the Live Imaging 3.1 software.

2.5. Tumor growth retardation study and immunohistology analysis

Female nude mice bearing H460 xenograft tumors (size 40–50 mm^2) were produced six days after subcutaneous injection of 5×10^6 NCI-H460 cells on the rear side of the back of mice. CytC or MPS-cytC in different NP formulations were i.v. injected in each respective treatment group (160 μg/kg, administered every other day). Tumor growth in the treated mice was monitored daily by measuring the diameters of tumor with calipers. The tumor volume was calculated as $(W2 \times L)/2$, where W is width and L is length. All mice were eventually sacrificed by cervical dislocation (after 22 days), and tumor tissues from mice were collected for immunohistological study. Serotological and hematological factors were also evaluated to assess toxicity of the various treatments (Table S1). Samples were fixed in a 10% formaldehyde solution for 2 h and were then washed with PBS. Tumor sections were prepared by washing tumor tissue with 70% ethanol, embedding the tumors in paraffin and deparaffinizing the samples in xylene. The sections were then rehydrated in ethanol and washed in phosphate-buffered saline (PBS). Prepared sections were incubated with a 1:200 dilution of anti-Caspase-3 rabbit serum for 2 h at 25 °C. HRP-labeled secondary antibody (Santa Cruz biotechnology Inc., CA) was added to the blots at a dilution of 1:500 for 20 min. The sections were co-stained with H&E stain and were observed with a microscope (Nikon Eclipse, Ti-U, Nikon instrument Inc. TX).

3. Results

3.1. NP size and charge characterization

Lipid nanoparticles (NPs) were produced using DOTAP, DOPE, and apolipoprotein (Fig. 1). Using a Nanosizer, we determined that a 2:1 DOTAP:DOPE molar ratio produced lipid NPs of optimal size (<100 nm) and highly positive charge (50–55 mV) in an aqueous environment. Nanoparticles consisting of DOTAP/DOPE and apolipoprotein were smaller (20–30 nm) and displayed a lower cationic charge of 25–28 mV, attributed to enhanced lipid compaction induced by the lipid binding structure of the apolipoprotein. Either MPS-GFP or MPS-cytC was admixed and attached to the formed NPs during a 1 h formulation step. Protein-loaded NPs were then PEGylated with a mixture of DSPE-PEG_{2000} and DSPE-PEG_{2000}-AA (1:1). The resulting final particles, henceforth designated as "MPS-GFP-NP" or "MPS-cytC-NP" in the text, exhibited a slightly reduced size (20–30 nm) and a dramatically decreased surface charge (2–3 mV). Nanoparticles produced without DSPE-PEG_{2000} were larger and tended to aggregate. All NPs used in subsequent

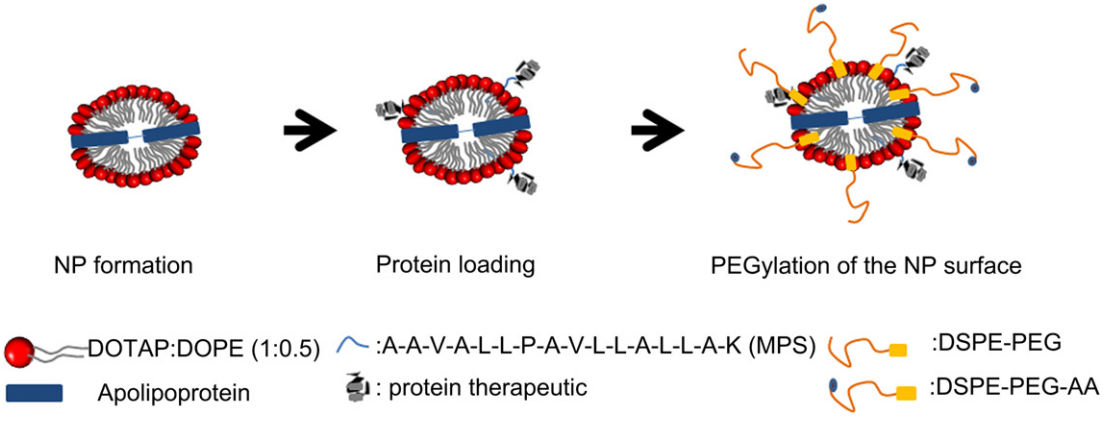

Fig. 1. Schematic picture depicting our NP manufacturing platform for protein delivery.