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Imaging and therapy of liver fibrosis using bioreducible polyethylenimine/siRNA complexes conjugated with N-acetylglucosamine as a targeting moiety

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ABSTRACT

Diagnosis and therapy of early stage liver fibrosis is very important for the treatment of fatal liver diseases. Here, we report on the targeted imaging and therapy of activated hepatic stellate cells (HSCs) and fibrotic liver tissue using N-acetylglucosamine (GlcNAc)- and indocyanine green (ICG)-conjugated PEI/siRNA complexes. The conjugation of a disulfide bond to PEI (PEI-D) was achieved by Michael addition. We modified PEI with N-acetylglucosamine (PEI-D-GlcNAc), which can specifically interact with desmin on activated HSCs, using the EDC coupling method. Confocal microscopic analysis showed that the PEI-D-GlcNAc/siRNA was internalized by HSCs upon interaction with surface desmin. *In vitro* western blot analysis confirmed that PEI-D-GlcNAc provided strong protein knock-down after transfection with TGFβ1siRNA into HSCs. After a tail vein injection of ICG-conjugated complexes, the PEI-D-GlcNAc-ICG/siRNA complex accumulated to a greater extent in the livers of fibrotic mice than in normal mice over an extended duration. Moreover, immunohistofluorescence analysis confirmed that the PEI-D-GlcNAc-ICG/siRNA complex specifically colocalized with HSCs, which are desmin-positive cells, in fibrotic liver tissues. *In vivo* TGFβ1siRNA delivery also resulted in superior protein knock-down when using the PEI-D-GlcNAc complex. These results demonstrate that the PEI-D-GlcNAc-ICG/TGFβ1siRNA complex is a useful tool for imaging and treatment of liver fibrosis.

1. Introduction

Small interfering ribonucleic acid (siRNA) delivery is a potential method for therapy of several diseases by knock-down of specific disease-related genes and proteins [1]. However, in addition to being degraded by enzymes, siRNA cannot penetrate cellular membranes [2]. To overcome these problems, many researchers have investigated systems for stable siRNA delivery to cells or organs using viral (e.g., adenovirus, lentivirus) [3–6] and nonviral (e.g., liposomes, cationic polymers, dendrimers) [7–11] vectors. Especially, siRNA delivery systems using bioreducible polymers are attractive because of their increased cell viability and transfection efficiency [12–15]. The disulfide bond in bioreducible polymers is easily reduced under intracellular conditions, which maintain a high concentration of glutathione. Thus, bioreducible gene delivery systems show good stability in the extracellular space and an efficient release of siRNA in the intracellular region.

Liver fibrosis is related to fatal liver diseases, such as liver cancer and liver cirrhosis. To avoid common liver diseases, diagnosis and therapy of liver fibrosis in its early stages is critical. Activated hepatic stellate cells (HSCs) play important roles in liver fibrosis. In the damaged liver, HSCs change their phenotype from a quiescent state to an activated state, inducing the secretion of cytokines and growth factors by hepatocytes and other non-parenchymal cells [16,17]. Activated HSCs also produce extracellular matrix (ECM) components, such as collagen, which contribute to liver fibrosis. Therefore, the targeting of activated HSCs and the delivery of genes or drugs to activated HSCs are key points of antifibrotic therapy.

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Recently, we discovered that polymers bearing N-acetylglucosamine (GlcNAc) have a strong avidity for vimentin and desmin on cell surfaces [18,19]. Based on these results, we reported a vimentin-mediated gene delivery system using GlcNAc-immobilized on polyethylenimine [20]. This GlcNAc-conjugated gene carrier system efficiently transfected genes into vimentin-positive cells. We also reported that an N-acetylglucosamine-bearing polymer, PVGlcNAc, could identify vimentin and desmin-positive cells in a population of liver cells [21]. The N-acetylglucosamine-bearing polymer interacted with vimentin-positive liver cells, such as sinusoidal endothelial cells, Kupffer cells, and HSCs. Specifically, activated HSCs expressed both vimentin and desmin more strongly than quiescent HSCs and other non-parenchymal cells. Therefore, we predicted that our GlcNAc-immobilized carrier system was specifically targeted to activated HSCs and thus regions of liver fibrosis.

Indocyanine green (ICG) a fluorescent dye that is approved by the Food and Drug Administration (FDA) and is broadly used for the imaging and diagnosing of diseases [22,23]. ICG has also been exploited to measure hepatic function by measuring its rate of retention in the blood [24]. However, the ICG fluorescence signal quickly disappears from the blood when ICG is injected alone. To obtain long-lasting ICG imaging, conjugation with nanomaterials is required. Polymers and phospholipids have been used to increase the stability of ICG in blood [25,26].

In this study, N-acetylglucosamine- and indocyanine green-conjugated nanocomplexes were designed for the efficient diagnosis and therapy of liver fibrosis. Low-molecular-weight polyethylenimine (MW 800) was conjugated to cystamine bisacrylamide to provide reducibility, and immobilization of ICG was used to enable the diagnosis of liver fibrosis. GlcNAc was immobilized to provide efficient gene delivery into activated HSCs via its interaction with surface vimentin and desmin. The size and distribution of the complexes were measured by transmission electron microscopy (TEM) and dynamic light scattering (DLS). In vitro uptake of the complexes was observed by confocal microscopy. Optical imaging of liver fibrosis by GlcNAC-immobilized complexes was performed using an IVIS Lumina imaging system. The therapeutic effect on liver fibrosis was confirmed by western blotting and histological analysis.

2. Materials and methods

2.1. Materials

Polyethylenimine (PEI) (0.8 KDa) was purchased from Sigma–Aldrich (Saint Louis, MO, USA). N,N′-cystamine bisacrylamide (CBA) was purchased from Polysciences (Warrington, PA, USA). Chitobiose was obtained from Megazyme (Wicklow, Ireland). 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), water-soluble carbodiimide [1-ethyl-3-3(-dimethylaminopropyl) carbodiimide hydrochloride (EDC)], and 1,4-dithiothreitol (DTT) were purchased from Sigma–Aldrich. Monoclonal mouse anti-TGF-β1 was obtained from R&D systems (Minneapolis, MN, USA). Monoclonal mouse anti-β-actin, monoclonal mouse anti-desmin, and monoclonal mouse anti-α-smooth muscle actin were purchased from Sigma Aldrich. Horseradish peroxidase (HRP)-conjugated mouse secondary antibody was obtained from Abcam (Cambridge, UK). 2-[7-[1,3-Dihydro-1,1-dimethyl-3-(4-sulfobutyl)-2H-benzo[e]indol-2-ylidene]-1,3,5-heptatrienyl]-1,1-dimethyl-3-[5-(3-sulfosuccinimidyl)oxycarbonylpentyl]-1H-benzo[e]indolium (ICG-Osu) was purchased from Dojindo Molecular Technologies, Inc. (Tokyo, Japan). Stellate cell medium was purchased from Sciencell Research Laboratories (Carlsbad, CA, USA).

2.2. Immobilization of disulfide bond, GlcNAc and ICG to PEI (PEI-D-GlcNAc-ICG)

Disulfide bond-conjugated PEI (PEI-D) was obtained from a Michael addition using CBA [27]. Briefly, 0.8 KDa PEI (10% aqueous methanol) and CBA (at a molar ratio of 1:2; reactive group of CBA versus primary amine group in PEI) were mixed at 55 °C under a nitrogen atmosphere for 24 h. Unreacted CBA was eliminated by addition of excess PEI. The purification of product was performed using a dialysis membrane (MWCO 3500). Immobilization of GlcNAc to PEI-D (PEI-D-GlcNAc) was achieved by the EDC coupling method as described in a previous report [20].

To conjugate ICG to PEI-D-GlcNAc (PEI-D-GlcNAc-ICG), ICG-Osu was dissolved in dimethyl sulfoxide (1 mg/ml). An equal weight of polymer (2 mg/ml in PBS) and ICG-Osu were mixed for 2 h at room temperature. Unreacted ICG-Osu was eliminated by centrifugation at 15,000 rpm. Immobilization of the disulfide bond and GlcNAc was determined by FT-IR (Bruker Optics IF66, Billerica, MA, USA) and ^{1}H NMR spectroscopy (JEOL Ltd, JLM-AL400, Tokyo, Japan). The conjugation of ICG was examined using UV-Vis spectroscopy (Beckman Coulter, DU-800, Brea, CA, USA). The synthesis scheme is presented in Supplemental Data 1.

2.3. Preparation and characterization of the complex

Various weight ratios of siRNA and PEI-D-GlcNAc were mixed to prepare the complexes. To confirm the presence of the disulfide bond, 50 mM DTT was mixed with the complex solution for 10 min at room temperature. The formation of the complex was confirmed by a gel retardation assay. Briefly, samples of the complexes were electrophoresed in Tris base, acetic acid, and ethylenediaminetetraacetic acid (EDTA) (TAE) solution through 100 V for 15 min using 1 wt % of agarose gel containing 0.1% ethidium bromide. To measure the size and zeta potential, the polymer (PEI-D-GlcNAc, PEI-D-GlcNAc-ICG) and siRNA (at a weight ratio of 20) were mixed in distilled water and incubated for 20 min at room temperature. The size and zeta potential of the complexes then were measured by dynamic light scattering (DLS, Otsuka Electronics, Osaka, Japan). The fluorescence intensity of ICG in the complexes after the addition of DTT was measured by a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelengths of 805 nm and 820 nm, respectively.

2.4. Cellular cytotoxicity

A 1×10^4 (cells/well) of rat hepatic stellate cells (Sciencell Research Laboratories, Carlsbad, CA, USA) were seeded into 96-well cell culture plates in stellate cell medium containing 10% FBS and penicillin streptomycin for 24 h. An MTT assay was performed to estimate the cell viability. Various concentrations of polymers were incubated with the cells for 24 h. The cell medium was replaced, and 10 μl of MTT (5 mg/ml in phosphate buffered saline (PBS)) solution was added to the cells for 4 h. After 4 h, the cells were washed, and formed formazan was dissolved in 100 μl of dimethyl sulfoxide (DMSO) for 15 min. The absorption was measured at 570 nm by microplate reader (SpectraMax M2, Molecular Devices).

2.5. Cellular uptake and interaction of the complexes with desmin

The cellular uptake and interaction of the complexes with desmin were observed by confocal microscopy. Briefly, rat HSCs were cultured in eight-well chamber slides at a density of 1×10^4 (cells/well) for 24 h before transfection. Fluorescein isothiocyanate (FITC)-conjugated siRNA and the synthesized polymers (PEI-D, PEI-D-GlcNAc) were incubated for 30 min at room temperature in stellate cell medium. The complex was then incubated with the cells for 4 h, and the cells were washed with PBS three times. The cells were fixed using 4% paraformaldehyde for 10 min. The cells were permeabilized with 0.1% Triton X-100 to stain intracellular desmin. These cells were blocked with 1% bovine serum albumin (BSA) for 30 min, incubated with rabbit polyclonal desmin antibodies (Sigma–Aldrich) for 2 h, and then incubated with Cy3-conjugated goat anti-mouse IgG antibodies as secondary antibodies (Jackson Immune Research Laboratory, West Grove, PA, USA) for 2 h at room temperature. The nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI) solution.

2.6. Preparation of fibrotic mice

Carbon tetrachloride (CCl_4) was used to produce hepatic fibrosis in mice. Briefly, CCl_4 was dissolved in olive oil (1:5), and 1 μl/μg body weight of CCl_4 solution was injected intraperitoneally into 5–6-week-old female Balbc/nu mice (SLC, Inc., Hamamatsu, Japan) twice a week for 5 weeks. All animal care and experiments were approved by the Animal Care Committees of the Korea Research Institute of Bioscience and Biotechnology (KRIBB).

2.7. Imaging and therapy of liver fibrosis

For the diagnosis and therapy of liver fibrosis, the PEI-D-GlcNAc-ICG/TGFβ1siRNA complex was injected into mice intravenously (200 μl), and the PEI-D-GlcNAc/TGFβ1siRNA complex was injected 3 days after the first injection. Complex- and ICG-injected mice were observed after 1 day and 2 days by IVIS® Lumina imaging system (Xenogen, Alameda, CA, USA) with an ICG filter set. The therapeutic effect of the PEI-D-GlcNAc-ICG/TGFβ1siRNA complex was confirmed by western blotting and H&E staining after 7 days after the first transfection. To perform ex vivo imaging, the mice were sacrificed, and the liver, spleen, and kidneys were observed with the imaging system 6 days after the injection of the complexes.

2.8. Western blotting

Proteins from cells and tissues were analyzed by western blotting. Cells and tissues were lysed in RIPA buffer (GeneDepot, Barker, TX, USA) and centrifuged at