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Quick nuclear transportation of siRNA and *in vivo* hepatic ApoB gene silencing with galactose-bearing polymeric carrier

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ABSTRACT

Since previous studies have linked the genetic mutations of Apolipoprotein B (ApoB) to the low density lipoprotein (LDL) cholesterol levels, it can be believed that the knockdown of ApoB by siRNA silencing is a useful method to reduce the cardiovascular disease. However, the spontaneous uptake of siRNA is hindered, and thus vectors are necessary to aid its transfer into the cells. Among the synthetic non-viral vectors, cationic polymers are extensively investigated as possible candidates for efficient and specific gene delivery, because they can be easily modified to get different set of properties.

Therefore, in this work a set of random copolymers with different molecular weight and composition were synthesized. These vectors present 2-(dimethylamino)ethyl methacrylate, as cationic monomer, and galactose units as liver-targeting moieties. From *in vitro* experiments, copolymers with monomer ratio and molecular weight about 0.1 and 80 kDa, respectively, showed adequate transfection capabilities and displaying good cell viability, independently of the nature of the saccharides units. However, in the *in vivo* experiments in C57BL/6 high-fat-fed mice, a better blood compatibility and protection against degradation leading to better transfection by the random copolymers bearing galactose units was confirmed.

1. Introduction

ApoB is a glycoprotein indispensable in the transport and receptor-mediated uptake of the LDL [Davidson et al., 2000]. The concentration of ApoB-containing lipoproteins has been correlated with atherosclerosis [Davidson et al., 2000], hypercholesterolemia [Seed et al., 1990], myocardial infarction [Sandkamp et al., 1990], and thrombosis [Nowak-Gottl et al., 1997]. Conversely, ApoB mutations, which lower its blood content, appear to be associated with reduced levels of atherosclerosis [Linton et al., 1993; Neuman et al., 2002; Tarugi et al., 2001; Schonfeld et al., 2003]. These observations have encouraged interest in the pharmacologic inhibition of ApoB. In small animal and non human primates, the knockdown of ApoB, by RNAi and antisense oligonucleotides, has been associated with low LDL cholesterol levels [Crooke et al., 2005; Zimmermann et al., 2006]. Therefore, for a broad range of patients, including the statin-intolerant, this approach can be useful to reduce the major risk factor for cardiovascular disease.

Since most of the plasma ApoB is made in the liver, the therapeutic nucleotides have to specifically reach the liver. Crooke et al. [Crooke et al., 2005] took advantage of the higher accumulation in the liver and kidney of the antisense oligonucleotides. Instead, Zimmermann et al. [Zimmermann et al., 2006] delivered siRNA in a liposomal formulation. In particular, Zimmermann et al. [Zimmermann et al., 2006] showed that intravenous injection of ApoB-liposome successfully targeted the liver to silence the ApoB gene, although they reported a significant elevation in serum transaminases indicative of hepatocellular necrosis at the effective dose.

Our study illustrates a new strategy to reduce the toxic effect of cationic carriers used as delivery vector for siRNA molecules which silenced ApoB gene. Since its discovery, the RNAi-mediated silencing in response to systemic delivery of siRNA, has been efficiently used in mammalian cells and it has reached several clinical trials [de Fougerolles et al., 2007; Whelan, 2005; Corey, 2007a, b]. However, siRNA direct delivery continues to be problematic because of their rapid degradation by nucleases, limited blood

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stability, poor cellular uptake, and nonspecific targeting [Stein, 1996; Urban-Klein et al., 2004; Katas and Alpar, 2006]. Therefore, vectors which can both protect and transport siRNA to target cells are required. The non-viral gene delivery vectors have many advantages over their viral counterparts, including the lack of specific immune response and the ability to deliver large pieces of nucleic acids [Wagner and Kloecker, 2006; Li and Huang, 2006; Mintzer and Simanek, 2009]. In the past decades, a wide range of non-viral gene delivery vectors such as liposomes [Chien et al., 2005], micelles [Itaka et al., 2003], and cationic polymers [Wagner et al., 1990] have been investigated. In particular, special attention has been given to the cationic polymers, which can be easily modified to fine tune their physiochemical properties. The presence of amino groups in the carrier chains confers a polycationic nature, which enable the polyanionic compounds such as nucleic acids to condense. Their positively charged character promotes the adsorptive endocytosis by cells, but on the other hand, it induces interaction with negatively charged serum proteins *in vivo*. Therefore, several modifications have been developed to prevent the interactions with the blood components. However, for specific delivery into liver cells, the use of cationic polymers with carbohydrate units seems the most promising modification. In fact, carbohydrates in cationic polysaccharides are thought to be involved in condensing DNA *via* hydrogen bonding, thus they can reduce the required excess of cationic charge and hence decrease the toxicity [Ahmed and Narain, 2011]. In addition, the carbohydrate moieties can interact with specific cellular receptors and may be useful as targeting agent. In our previous study, we synthesized a polyethyleneimine(PEI)-pullulan conjugate as a liver-targeting carrier [Kang et al., 2010] and we demonstrated that the PEI-pullulan/siRNA complex rapidly accumulated in the liver after intravenous injection, in contrast with PEI/siRNA complex, which showed no targeting capabilities.

In the present work, random copolymers of 2-(dimethylamino) ethyl methacrylate (DM) and 6-*O*-vinyl adipoyl galactose or 6-*O*-vinyl adipoyl glucose were synthesized. The radical copolymerization of these monomers provides a variety of carrier polymers with different charge density, different targeting sugar moieties, and different molecular weight. The DM was selected because of its polycationic nature and its ability to condense plasmid DNA in small particles with good transfection efficiency [van de Wetering et al., 1997, 1998]. The stability, toxicity, and uptake by hepatocytes (NMuLi cells) of the carrier/siRNA complexes were studied. Furthermore, the time course of intracellular localization of the complexes was observed under a fluorescent confocal laser scanning microscope. Finally, *in vivo* suppression of ApoB mRNA was investigated after intravenous injection of carrier/siRNA complexes.

2. Materials and method

2-(Dimethyl amino) ethyl methacrylate stabilized with MEHQ (DMAEMA, Mw 157.21. (Wako Pure Chemical Industries, Ltd. Japan) was used after purification. 6-*O*-vinyl adipoyl galactose (Gal) and 6-*O*-vinyl adipoyl glucose (Glu) were gifts from Green Products Laboratory LLC (Windham, ME USA). Azobis(isobutyronitrile) (AIBN; Wako, Oska Japan), dimethyl sulfoxide (DMSO; Wako, Osaka Japan) were used as received.

The murine normal liver epithelial cell line (NMuLi) was provided by ATCC (Tokyo, Japan). They were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM-HG; Gibco, Carlsbad, CA) supplemented with 10% bovine calf serum (Gibco-Invitrogen, Carlsbad, CA), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco-Invitrogen, Carlsbad, CA).

The siRNA (indicated as) used in this study with the following sequences were synthetized by Gene Design (Osaka, Japan) and denoted as siApoB, sense: 5′-GUCAUCACACUGAAUACCAAUdTdT-3′, and antisense: 5′-AUUGGUAUUCAGUGUGAUGACACdTdT-3.

2.1. Synthesis of a siRNA carrier

Random copolymers were prepared by radical polymerization from the monomers (Fig. 1(a)-(c)) using AIBN as initiator. We systemically varied the monomer composition in feed (Table 1, total monomer amount was 4 mmol) in 6.4 mL DMSO. 0.5 mol% AIBN were added into 1.6 mL of DMSO, in another dried glass tube, and subsequently mixed with the solution of the two monomers in DMSO. The mixture was cooled immediately with liquid N_2, and after three freeze-thaw cycles, it was incubated at 60 °C for 4 h. The polymerization reaction was terminated by cooling in an ice water bath. The mixture was diluted with cold water and the resulting copolymers were purified using dialysis (Spectra/Pore membrane, cut-off molecular weight = 1×10^3 Da) in distilled water. The remaining solution was lyophilized. The detailed copolymers used in this study are listed in Table 1.

2.2. Copolymers characterization

^{1}H NMR spectra were recorded on a 300-MHz NMR spectrometer (Gemini2000/300; Varian Inc., CA) with a sample concentration of 8 mg/800 μL in deuterium oxide. Size exclusion chromatography (SEC) analysis was carried out using Shimadzu Gel Permeation Chromatography System apparatus equipped with a refractive index (RI) and UV detectors.

The 1 mg/100 μL of sample were dissolved in eluent (with 55% H_2O, 45% acetonitrile, and 0.1% trifluoroacetic acid), filtered over 0.2 μm, and placed in a thermally controlled sample holder and injected on the 2 columns (TSKgel G6000PWXL and G3000PWXL, Toso, Tokyo, Japan) connected in series and remained at 40 °C with a temperature control module.

The sugar unit composition of each polymer was calculated by the elemental analysis of C, N, O.

2.3. Gel retardation assay and preparation of polyplexes

Copolymer/siApoB complexes differing in charge ratio but having the same siApoB concentration (100 pmol in 3 μL) were prepared as follows. Different volumes of carrier stock solutions were added to a fixed volume of siApoB stock solution in one step. The dispersion was vortexed for 10 s. The polyplexes were allowed to equilibrate for 30 min at room temperature before use. The polyplexes were applied to a 19% acrylamide gel for 60 min at 150 V in 1 × tris-acetate-EDTA (TAE) buffer. The retardation of the complexes was confirmed by ethidium bromide staining.

2.4. Particle size and ζ-potential measurements

Complexes were formed as described above before performing measurements at various N/P ratios in deionized water. Dynamic light scattering measurements on polyplexes were carried out on a Malvern Zetasizer Nano ZS (Malvern, Worcestershire, U.K.) at 25 °C and at an angle of 173°. The incident beam was a HeNe laser beam (633 nm). The z-average hydrodynamic diameter measurements were carried out at an siApoB concentration of 0.1% (w/v). The ζ potential measurements were carried out at an siApoB concentration of 20 μg/mL in a 0.01 M NaCl solution.

2.5. In vitro transfection and toxicity

Transfection and toxicity studies were performed in NMuLi cells. Toxicity was carried out in 96-well plates at a density of 1×10^4 cells/well. Complexes of a range of N/P ratios from 0.375