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Development of antibody-siRNA conjugate targeted to cardiac and skeletal muscles

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ABSTRACT

Despite considerable efforts to develop efficient carriers, the major target organ of short-interfering RNAs (siRNAs) remains limited to the liver. Expanding the application outside the liver is required to increase the value of siRNAs. Here we report on a novel platform targeted to muscular organs by conjugation of siRNAs with anti-CD71 Fab′ fragment. This conjugate showed durable gene-silencing in the heart and skeletal muscle for one month after intravenous administration in normal mice. In particular, 1 μg siRNA conjugate showed significant gene-silencing in the gastrocnemius when injected intramuscularly. In a mouse model of peripheral artery disease, the treatment with *myostatin*-targeting siRNA conjugate by intramuscular injection resulted in significant silencing of *myostatin* and hypertrophy of the gastrocnemius, which was translated into the recovery of running performance. These data demonstrate the utility of antibody conjugation for siRNA delivery and the therapeutic potential for muscular diseases.

1. Introduction

Systemic short-interfering RNA (siRNA) delivery has remained a major obstacle hindering the development of RNA interference (RNAi) therapeutics. Significant progress has been made in recent years using *N*-acetylgalactosamine (GalNAc) as a targeting ligand for liver specific asialoglycoprotein receptor [1,2]. GalNAc-siRNA conjugates are able to successfully silence the liver-expressed genes. Accordingly, clinical trials have been initiated for treating hemophilia in phase I [3] and transthyretin-mediated amyloidosis in phase III [4] using GalNAc-siRNA conjugate targeting antithrombin and transthyretin, respectively. Utilization of the other ligand-receptor pair on siRNA conjugates is expected to expand the application of RNAi therapeutics outside the liver; however, no remarkable ligands have as yet been reported.

Transferrin (Tf) is an iron-binding glycoprotein that transports ferric ions into cells via the Tf receptor, CD71 [5]. CD71 is ubiquitously expressed in normal cells, including the endothelium of brain capillaries, hepatocytes, Kupffer cells, and tubules of the kidney [6]. Endothelial cells on brain vasculature carry iron into the central nervous system

Abbreviations: siRNA, small interfering RNA; Tf, transferrin; ApoB, apolipoprotein B; HPRT, hypoxanthine-guanine phosphoribosyltransferase; ACTB, β-actin; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; Rplp0, ribosomal protein large P0; GalNAc, *N*-acetylgalactosamine; *i.v.*, intravenous administration; *i.p.*, intraperitoneal administration; *s.c.*, subcutaneous administration; SEC-HPLC, Size Exclusion high-performance liquid chromatography; ADC, antibody drug conjugate; PAD, lower extremity peripheral artery disease; FAL, femoral artery ligation; L-PHA, phytohaemagglutinin-L_4; PBS, phosphate buffer saline; s.d., standard deviation; PNA, peptide nucleic acid.

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via CD71-mediated transcytosis [7], which has been used to deliver pharmacological agents across the blood brain barrier [8,9]. CD71 is particularly abundant in maturing erythroid cells because their requirement for iron in hemoglobin is the greatest [10]. Skeletal and cardiac myocytes are also rich in CD71 because of their requirement for considerable myoglobin synthesis [11]. In addition, the ferric ions play a key role in cellular growth and proliferation [12,13]. Therefore, the expression of CD71 is elevated in proliferating cells because of the need for iron [14], and many anti-CD71-cytotoxin conjugates have reportedly been used in cancer therapy [15].

The internalization of CD71 has been extensively studied [16]. Cells constitutively internalize CD71 by clathrin-dependent endocytosis, and transport Tf into early endosomes. Tf is then dissociated from CD71 at acidic endosomal pH, and CD71 subsequently recycles to the cell surface. This turnover occurs rapidly and repeatedly at >100 times per molecule [17]; therefore, CD71-mediated endocytosis has been utilized to deliver not only cytotoxins, but also nucleic acids into cells. To develop CD71-targeted nucleic acids, numerous carriers have been reported. Wagner et al. conjugated Tf with a polycation as a DNA-binding domain [18]. The Tf-polycation/plasmid complex was taken up by the eukaryotic cells, and an efficient luciferase expression was accomplished. Liang et al. conjugated Tf with peptide nucleic acid (PNA) that has an affinity for plasmid DNA, and the resultant Tf/PNA/plasmid complex showed transfection activity against myogenic cells [19]. Anti-CD71 scFv-modified cationic liposomes containing *p53* expression-plasmid, SGT-53, are currently undergoing phase II clinical trials for the treatment of tumors [20]. For siRNA delivery, Tf modified cyclodextrin/siRNA complex was explored in a phase I trial for the treatment of tumors [21]. These vehicles commonly contain cationic compounds to bind nucleic acids, thereby forming predominantly submicrometer-sized particles (nanoparticles). These nanoparticles can be exuded and accumulated in a tumor by the enhanced permeability and retention (EPR) effect [22], as some tumors are characterized as a well-perfused tissue containing immature vasculatures. However, the action of such complexed nucleic acids on the other organs might be hindered because of the low extravasation across the endothelium of normal vasculature.

In contrast, a distinguished feature of siRNA conjugate is its applicability to subcutaneous administration, as was demonstrated by GalNAc-siRNA conjugate [1]. The small molecular size might allow efficient penetration across the tissues, and the chemical modifications on siRNA may provide protection from degradation before reaching the target organ. Therefore, we conjugated anti-CD71 antibody Fab′ fragment directly with chemically stabilized siRNA to develop a minimum-sized delivery platform, which escapes from the hindrance caused by the size of nanoparticles and can fully exploit the CD71-targeting potentials. Here, we demonstrate that anti-CD71 Fab′ conjugated siRNA targeting hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) (anti-CD71 siHPRT) causes robust and durable silencing effects in muscular organs. Furthermore, we explored the silencing of the muscle-expressed gene, *myostatin*. Myostatin is a transforming growth factor-β superfamily protein that negatively regulates muscle mass. Therefore, myostatin inhibition therapy and the resultant muscle hypertrophy are expected for muscle disorder or metabolic diseases therapy [23]. To evaluate the versatility of anti-CD71 siRNA conjugates in the study of skeletal muscle myopathy, we applied anti-CD71 Fab′ conjugated siRNA targeting *myostatin* (anti-CD71 siMSTN) for a mouse model of peripheral artery disease (PAD) with femoral artery ligation (FAL). PAD is one of the major muscular diseases caused by extensive atherosclerosis and thrombosis in the artery to the lower extremities that leads to skeletal muscle ischemia and myopathy associated with walking limitation because of leg pain. The loss of muscle mass in the lower extremity is a hallmark in patients with PAD [24,25]. The incidence of walking disorder (intermittent claudication) was reportedly 1 per 1000 people in a population per year [26]. Here, we demonstrated that the antibody-siRNA conjugate was an effective platform to study muscular diseases.

2. Materials and methods

2.1. Materials

Rat monoclonal anti-mouse CD71 (clone R17 217.1.3) and isotype control IgG_2a (clone 2 A3) were purchased from BioXcell (West Lebanon, NH). The sequences for the sense and antisense strands of *ApoB*, *HPRT*, *myostatin*, and the negative control siRNAs are as follows: 1) siApoB sense: 5′-GgAaUcUuAuAuUuGaUcCaA-$(CH_2)_6NH_2$-3′; antisense: 5′-puUgGaUcAaAuAuAaGaUuCcsCsu-3′; 2) siHPRT sense: 5′-UcCuAuGaCuGuAgAuUuUaU-$(CH_2)_6NH_2$-3′; antisense: 5′-paUaAaAuCuAcAgUcAuAgGasAsu-3′; 3) siMSTN sense: 5′-GaGuAuGcUcUaGuAaCgUaU-$(CH_2)_6NH_2$-3′; antisense: 5′-aUaCgUuAcUaGaGcAuAcUcsAsa-3′; 4) siNC sense: 5′-UgUaAuAaCcAuAuCuAcCuU-$(CH_2)_6NH_2$-3′; antisense: 5′-aAgGuAgAuAuGgUuAuUaCasAsa-3′. 2′-*O*-methyl–modified nucleotides are in lowercase, 2′-fluoro–modified nucleotides are underlined, and phosphates and phosphorothioate linkages are indicated as "p" and "s," respectively. The sense strand with a C6 amino linker at the 3′-end and antisense strand of each siRNA were synthesized by DNA/RNA synthesizer. GalNAc-siApoB conjugate were synthesized according to the previous reports [1]. Thiol reactive siRNAs were prepared by reaction of the amino group of C6 amino linker with bifunctional crosslinkers; *N*-succinimidyl 3-maleimidopropionate (BMPS) (Acme Bioscience, Palo Alto, CA), *N*-succinimidyl 4-maleimidobutyrate (GMBS) (DOJIN, Japan), *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Thermo Fisher Scientific Inc., Waltham, MA), *N*-maleimidocaproyl-valyl-citrullyl-*p*-aminobenzylcarbamate *p*-nitrophenyl ester (mc-Val-Cit-PABC-PNP), or *N*-succinimidyl 4-methyl-4-(2-pyridyldithio) pentanate (Synchem, Elk Grove Village, IL). 4-methyl-4-(2-pyridyldithio) pentanate-modified siRNAs were reduced with 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd., Japan), and then a maleimide group was formed by the reaction with GMBS. This linker is described as dimethyl SS (DMSS) linker. Unless otherwise noted, BMPS was used to form the linker between siRNAs and antibodies, which we refer to as maleimide linker. All the reactions were monitored by high performance liquid chromatography-mass spectrometry (HPLC-MS), and excess reagents were removed using Amicon Ultra ultrafiltration devices (MWCO 3K, Millipore). Sense and antisense strands were annealed and used directly for the antibody conjugation.

2.2. Conjugation

$F(ab')_2$ fragments were prepared by conventional pepsin digestion of antibodies and were reduced with cysteamine to generate Fab′ fragments [27], which contain two thiol groups for use in conjugation [28]. Monovalent RI7 217 antibody is known to have binding affinity for CD71 without affecting cell proliferation [29]. After purification using a Sephadex G-25 column, Fab′ fractions were mixed with maleimide-modified or (2-pyridyldithio) pentanate-modified siRNA (Fig. 1A). Reactions were monitored using size-exclusion chromatography (SEC)-HPLC with TSKgel G2000SWxL (7.8 mm × 300 mm, TOSOH) with 20 mM phosphate-300 mM NaCl buffer (pH 7.0). After overnight incubation at room temperature, siRNA-Fab′ conjugates were separated from unreacted Fab′ and siRNAs using preparative SEC-HPLC. After concentration with an Amicon Ultra ultrafiltration device (MWCO 10 kDa), the concentrations of siRNA were quantified by a Quant-it RiboGreen RNA assay kit (Life technologies, Carlsbad, CA), and the concentrations of Fab′ were determined using ATTO-TAG FQ Derivatization Reagent (FQ; 3-(2-Furoyl) quinoline-2-carboxaldehyde) (Invitrogen, Carlsbad, CA) comparing their Fab′ as standards. The concentration and the weight of conjugates in the following studies are indicated as those of siRNA. Plasma siRNAs were extracted with Trizol and were then reverse transcribed by a TaqMan MicroRNA Reverse Transcription Kit (Life technologies, Carlsbad, CA) according to the manufacturer's instruction.