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Size-dependent specific targeting and efficient gene silencing in peritoneal macrophages using a pH-sensitive cationic liposomal siRNA carrier

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ABSTRACT

Macrophages are key contributors to various inflammatory diseases. Therefore, the development of an efficient *in vivo* short interference RNA (siRNA) system that can be delivered to macrophages represents a novel treatment strategy for addressing these disorders. It was recently revealed that peritoneal macrophages (PEMs) are involved in several diseases including ovarian cancer, and are now recognized as a promising drug target. We report herein on the use of pH-sensitive cationic YSK05-MENDs as siRNA carriers and on the impact of both the size of the YSK05-MENDs and their administration routes for the efficient targeting PEMs to achieve a high level of gene silencing activity. The size of the YSK05-MENDs had a dramatic effect on their specificity for PEMs when administered intravenously, but not for intraperitoneal injection. Also, significant gene silencing was achieved by an intraperitoneal administration of the YSK05-MEND at a dose in the single digit μg/kg range. To our knowledge, this is the most efficacious method for siRNA delivery for gene silencing in PEMs *in vivo* reported to date. These findings enabled us to investigate the complex function of PEMs through several gene silencing simultaneously.

1. Introduction

Since the discovery of RNA interference (RNAi) (Fire et al., 1998), the mechanism has become essential in terms of investigating the function of genes of interest. The effectors of RNAi are short interference RNA (siRNA) duplexes (20–25 nucleotides), which specifically degrade target mRNAs depending on their sequences. As siRNA can theoretically target most genes and can be designed and synthesized rapidly, applications of siRNAs for clinical use are predicted. However, the physicochemical properties of siRNAs, such as a high molecular weight (~13,000 Da), their hydrophilic nature and highly anionic charge, prevent passive diffusion across the plasma membrane of cells. Moreover, the low stability of siRNAs in an *in vivo* environment also makes their *in vivo* application difficult. Therefore, the development of efficient siRNA delivery methods, including formulations and routes of administration are essential for realizing RNAi medicine.

To date, various siRNA delivery systems, such as lipid, peptide and polymer based formulations, have been developed (Davis et al., 2010; Kim et al., 2010; Kumar et al., 2007; Love et al., 2010; Wooddell et al., 2013; Zimmermann et al., 2006). Lipid nanoparticles (LNPs) are known as the most advanced siRNA delivery technology for targeting hepatocytes (Jayaraman et al., 2012; Semple et al., 2010). These technologies recently achieved efficacious gene silencing in hepatocytes at a dose of ~0.01 mg/kg. This efficient delivery leads to the testing of LNPs in clinical trials.

Antibody and peptide based siRNA delivery carriers have been developed and succeeded in inhibiting viral replication and cytokine induction. Antibody modified liposomes containing siRNA have been reported to prevent the progression of colitis and HIV (Kim et al., 2010; Peer et al., 2008). Moreover, the intravenous administration of LNPs resulted in gene silencing in peritoneal macrophages (PEMs) and inflammatory monocytes in mice and non-human primates (Novobrantseva et al., 2012). However, the siRNA dose required to achieve this is significantly

Abbreviations: *t*-BuOH, tertiary butanol; chol, cholesterol; DC, dendritic cell; DMG, 1,2-dimirystoyl-sn-3-glycerol; LNP, lipid nanoparticle; MEND, multifunctional envelope-type nano device; PEG, polyethylene glycol; PEM, peritoneal macrophage; PLK1, polo-like kinase 1; RNAi, RNA interference; siRNA, short interference RNA.

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higher (≥0.5 mg/kg) compared to the hepatocyte-targeting system (~0.01 mg/kg) in an intravenous injection. In addition, the siRNA dose is high (≥0.2 mg/kg) even in an intraperitoneal injection to target PEMs (He et al., 2013; Lundberg et al., 2012). It is also noteworthy that the relationship between the physicochemical properties of the delivery carrier and their activity has not been investigated. PEMs are known to be involved in septic shock, peritoneal endometriosis and ovarian cancer (Naora, 2014; Pirdel and Pirdel, 2014). It has also been recently revealed that PEMs are a physically and functionally heterogeneous mass (Ghosn et al., 2010), and the GATA6 gene expression is essential for PEMs to exist in the peritoneal cavity (Okabe and Medzhitov, 2014). However, detailed studies of the functions and significance of PEMs are currently underway. Thus, we focused our attention on PEM targeting in this study.

We previously developed a multifunctional envelope-type nano device (MEND) containing a pH-sensitive cationic lipid, YSK05 (referred to as a YSK05-MEND) as a carrier for siRNA delivery (Hatakeyama et al., 2014; Hayashi et al., 2014; Sato et al., 2012; Watanabe et al., 2014). Here, to achieve efficient silencing, we focused on the impact of the size of the YSK05-MENDs and their administration route. In the case of intravenous administration, we revealed that the size of the particle was important in achieving specific-targeting for PEMs. It should also be noted that significant gene silencing was achieved via intraperitoneal administration at a dose in the single digit μg/kg range. This low dose enabled us to easily investigate the function of PEMs by modulating the expression of genes of interest.

2. Materials and methods

2.1. Materials

Anti-CD45 siRNA (siCD45, sense: 5′-**cuggcu** GAA **uuuc**AG AG**c** AT*T-3′; antisense: 5′-UGC UCU GAA AUU **c**AG C**c**A GT*T-3′, 2′-F-modified nucleotides are denoted in bold lower case letters and phosphorothioate linkages are represented by asterisks), anti-polo-like kinase 1 (PLK1) siRNA (siPLK1, sense: 5′-AGA uCA CCC uCC UUA AAu AUU-3′; antisense: 5′-UAU UUA AGG AGG GUG AuC UUU-3′, 2′-OMe-modified nucleotides are in lower case.) and anti-luciferase siRNA (siGL4, sense: 5′-CCG UCG UCU UCG UGA GCA ATT-3′; antisense: 5′-UUG CUC ACG AAU ACG ACH GTT-3′) were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan). YSK05 (1-methyl-4,4-bis(((9*Z*,12*Z*)-octadeca-9,12-dien-1-yl)oxy)piperidine) was synthesized as previously described (Sato et al., 2012). Cholesterol (chol) was purchased from Sigma–Aldrich (St. Louis, MO). 1,2-Dimirystoyl-*sn*-3-glycero methoxypolyethyleneglycol 2000 ether (PEG–DMG) was purchased from the NOF Corporation (Tokyo, Japan). Ribogreen was purchased from Molecular Probes (Eugene, OR, USA). DiD and TRIZOL reagent were purchased from Invitrogen (Carlsbad, CA, USA). All of the monoclonal antibodies (mAb) used in this study were purchased from BioLegend.

2.2. Experimental animal

ICR mice, four weeks old, were purchased from Japan SLC (Shizuoka, Japan). The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the guidelines for the care and use of laboratory animals.

2.3. Preparation of the MENDs

YSK05-MENDs were prepared as previously reported (Watanabe et al., 2014). Briefly, 2100 nmol of YSK05, 900 nmol chol and 6–90 nmol PEG–DMG (corresponding to 0.2–3 mol% of total lipid) were dissolved in 400 μL of 90% (v/v) aqueous tertiary butanol (*t*-BuOH). When the fluorescence was incorporated into the YSK-MENDs, 0.001 mol% (of the total lipid) of DiD was added to the lipid solution. Two hundred microliters of 80 μg siRNA solution was gradually added to the lipid solution under vigorous mixing. The siRNA-lipid solution was then gradually added to 2 mL of 20 mM citrate buffer (pH 4.0) under vigorous mixing. Finally, ultrafiltration was carried out for the removal of *t*-BuOH, adjustment of pH at 7.4, and the concentration of the YSK05-MENDs.

2.4. Physicochemical characterization of the MENDs

The size and ζ-potential of the YSK-MENDs were measured by a Zetasizer Nano ZS ZEN3600 instrument (Malvern Instruments, Worchestershire, UK). The encapsulation efficiency and recovery ratio of the siRNAs were measured by a RiboGreen assay as previously described (Sato et al., 2012).

2.5. Quantification of mRNA expression

Approximately 30 mg of liver and spleen tissues were homogenized using a Precellys 24 (Birthin technologies, France) in 500 μL of TRIZOL reagent. Total RNA was extracted according to manufacturer's protocol. The total RNA (1 μg) was reverse transcribed using a high capacity RNA-to-cDNA kit (ABI) according to manufacturer's protocol. A quantitative PCR analysis was performed on 2 ng cDNA using Fast SYBR Green Master Mix (ABI) and Lightcycler 480 system II (Roche). All reactions were performed at a volume of 15 μL. The primers for mouse Gapdh were (forward) 5′-AGC AAG GAC ACT GAG CAA G-3′ and (reverse) 5′-TAG GCC CCT CCT GTT ATT ATG-3′ and for mouse Ifit1 were (forward) 5′-AGA GAG CAG AGA GTC AAG G-3′ and (reverse) 5′-CAG CTG AAG CAG ATT CTC-3′.

2.6. Preparation of cell suspensions

Peritoneal cells were harvested by injecting 5 mL of cold PBS(−) into the peritoneal cavity. The spleens were disrupted and resuspended to obtain single cell suspensions. Red blood cells were lysed using RBC lysing buffer (Sigma–Aldrich). Cell pellets were resuspended by FACS buffer, which contains 0.5% (w/v) bovine serum albumin and 0.02% (w/v) sodium azide in PBS(−).

2.7. Flow cytometry

Cell suspensions ($0.5–1 \times 10^6$ cells/mL) were pre-incubated with purified anti-CD16/32 mAb (Clone 93) to block FcγRII/RIII receptors for 10 min and stained on ice for 30 min with the following fluorochrome-conjugated mAb: FITC-labeled anti-mouse F4/80 (Clone BM8), mouse Ly-6G (Clone 1A8); PE-labeled anti-mouse/human CD11b (Clone M1/70); PerCP-labeled anti-mouse CD11c (Clone N418); APC-labeled anti-mouse CD45 (Clone 30-F11). Cells were then washed by FACS buffer for 3 times. After washing, the stained cells were measured using FACSCalibur (Becton Dickinson) and analyzed using Cell Quest software (Becton Dickinson).

2.8. Animal treatment

For intravenous injection, ICR mice were injected with a dose from 1 to 2 mg/kg of the siRNA formulated in YSK05-MENDs. Liver and spleen tissues were collected at 4 h after the siRNA treatment, intraperitoneal and splenic cells were harvested 24 or 72 h after siRNA treatment.