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Systemic delivery of siRNA to tumors using a lipid nanoparticle containing a tumor-specific cleavable PEG-lipid

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ABSTRACT

Previously, we developed a multifunctional envelope-type nano device (MEND) for efficient delivery of nucleic acids. For tumor delivery of a MEND, PEGylation is a useful method, which confers a longer systemic circulation and tumor accumulation via the enhanced permeability and retention (EPR) effect. However, PEGylation inhibits cellular uptake and subsequent endosomal escape. To overcome this, we developed a PEG-peptide-DOPE (PPD) that is cleaved in a matrix metalloproteinase (MMP)-rich environment. In this study, we report on the systemic delivery of siRNA to tumors by employing a MEND that is modified with PPD (PPD-MEND). An in vitro study revealed that PPD modification accelerated both cellular uptake and endosomal escape, compared to a conventional PEG modified MEND. To balance both systemic stability and efficient activity, PPD-MEND was further co-modified with PEG-DSPE. As a result, the systemic administration of the optimized PPD-MEND resulted in an approximately 70% silencing activity in tumors, compared to non-treatment. Finally, a safety evaluation showed that the PPD-MEND showed no hepatotoxicity and innate immune stimulation. Furthermore, in a DNA microarray analysis in liver and spleen tissue, less gene alternation was found for the PPD-MEND compared to that for the PEG-unmodified MEND due to less accumulation in liver and spleen.

1. Introduction

The passive tumor targeting of liposomes is achieved through the enhanced permeability and retention (EPR) effect [1,2]. To produce liposomes that have a long circulation time, they are generally modified by poly(ethylene glycol) (PEG) and the product is referred to as a "stealth liposome" [3,4]. Stealth liposomes are used in clinical applications to deliver doxorubicin (Doxil) in the treatment of the acquired immunodeficiency syndrome (AIDS)-related Koposi's sarcoma [5].

However, PEGylation hampers in vivo applications of nanoparticles that are intended for use for the delivery of nucleic acids, such as plasmid DNA (pDNA) and small interfering RNA (siRNA). From the view point of nucleic acid delivery, it is necessary that nucleic acids arrive at an intracellular organelle for successful functioning (e.g. cytosol for siRNA, nucleus for pDNA) [6]. However, PEGylation inhibits the intracellular trafficking of nanoparticles, especially in cellular uptake and subsequent endosomal escape leading to significant loss of activity [7,8], which is referred to as the "PEG dilemma" [9].

To solve this, cleavable PEG systems that are removed in response to intracellular environments such as the low pH in

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endosomes/lysosomes and thiolytical molecules have been proposed [10–15]. Specific environments in tumor tissues have been also utilized as a trigger for PEG cleavage [16–20]. We constructed a tumor specific PEG system in which the PEG is removed by cleavage in the presence of a matrix metalloproteinase (MMP) [17]. MMPs are abundantly distributed in tumor tissue and play a key role in progress and metastasis [21]. A PEG-peptide-dioleoylphosphatidyl ethanolamine (DOPE) ternary conjugate (PPD) containing a peptide sequence that is sensitive to MMP-2 was synthesized. We recently developed a multifunctional envelope-type nano device (MEND), in which nucleic acids are condensed using a polycation to form a core particle that is encapsulated in a lipid envelope for use as a novel non-viral nucleic acid delivery system [22,23]. PPD modified MEND encapsulating pDNA showed enhanced transfection activity in tumors after systemic administration in tumor-bearing mice compared to conventional PEG modified MEND [17].

In the present study, we report on the systemic delivery of siRNA to tumors using a PPD modified MEND. A schematic diagram of the strategy employed is shown in Fig. 1. We tested whether PPD modification improved the intracellular trafficking of the MEND compared to conventional PEG-modified MEND in in vitro experiments. We then optimized the PPD modified MEND for in vivo siRNA delivery through the optimization of PPD modification, in which a combination of PEG-DSPE was used to achieve stability in systemic circulation and activity in tumor tissue. We evaluated the PPD modified MEND for its safety in cytokine studies and using a DNA microarray in the form of toxicogenomics studies [24].

2. Material and methods

2.1. Materials

Anti-luciferase siRNA (21-mer, 5′-GCGCUGCUGGUGCCAACCCTT-3′, 5′-GGGUUGGCACCAGCAGCAGCGCTT-3′), anti-green fluorescent protein (GFP) siRNA (21-mer, 5′-GCUGACCCUGAAGUUCAUCTT-3′, GAUGAACUUCAGGGUCAGCTT-3′) and the matrix metalloproteinase cleavable peptide (sequence: GGGVPLSLYSGGGG) were obtained from Thermo Electron GmbH (Ulm, Germany). DOPE, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), cholesterol, distearoyl-*sn*-glycero-3-phoshoethanolamine-N-[methoxy (polyethylene glycol)-2000] (PEG_{2k}-DSPE), PEG_{5k}-DSPE, 7-nitrobenz-2-oxa-1, 3-diazole labeled DOPE (NBD-DOPE) and rhodamine labeled DOPE (Rho-DOPE) were purchased from AVANTI Polar Lipids (Alabaster, AL, USA). Stearyl octaarginine (STR-R8) was obtained from KURABO INDUSTRIES (Osaka, Japan). PEG-peptide-DOPE (PPD) was synthesized as described previously [17]. RiboGreen and Lysotracker Green were purchased from Molecular Probes (Eugene, OR, USA). Alexa488-tagged siRNA and Alelx546-tagged siRNA was purchased from QIAGEN (Hilden, Germany). Luciferase assay reagents and reporter lysis buffer were obtained from Promega (Madison, WI, USA). [^{3}H] cholesteryl hexadecyl ether (CHE), Soluene-350 and Hionic Fluor were purchased from Perkin–Elmer Life Sciences Japan (Tokyo, Japan). ELISA assay kits of Quantikine Immunoassay mouse IL-6 and TNF-α were purchased from R&D systems (Minneapolis, MN, USA). Transaminase CII-test WAKO was obtained from Wako (Osaka, Japan).

2.2. Experimental animals

Male ICR mice (5–6 weeks old) and male BALB/c nude mice (5–6 weeks old) were purchased from CLEA (Tokyo, Japan). Tumor-bearing mice were prepared by the subcutaneous injection of male BALB/c nude mice with HT1080-luc cells (10^6 cells/mouse). All in vivo experiments were approved by the Institutional Animal Care and Use Committee.

2.3. Cell culture

HT1080 cells stably expressing luciferase (HT1080-luc) were cultured in cell-culture dishes (Corning) containing culture medium supplemented with 10% fetal

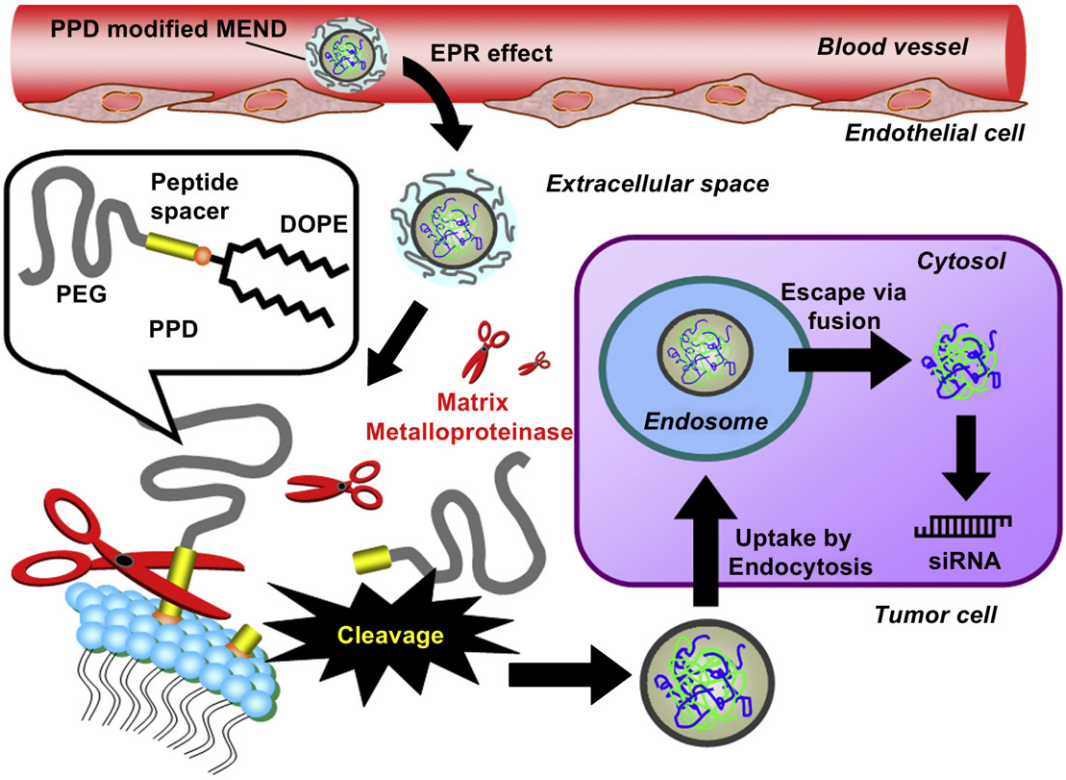

Fig. 1. A schematic diagram of the strategy for the systemic delivery of siRNA to tumors by the PPD modified MEND. PPD modified MEND accumulates in tumor via the EPR effect. MMP is abundantly secreted from tumor cells, which digests the peptide in the PPD. The PEG is then detached from the surface of the MEND, which allows MEND to associate with the tumor cell surface, followed by cellular uptake by endocytosis. The MEND escapes from endosome via membrane fusion, and finally the siRNA complex is delivered into the cytosol.