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Anti-angiogenic therapy via cationic liposome-mediated systemic siRNA delivery

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ABSTRACT

siRNA has been touted as a therapeutic molecule against genetic diseases, which include cancers. But several challenging issues remain in order to achieve efficient systemic siRNA delivery and a sufficient therapeutic effect for siRNA *in vivo*. Cationic liposome shows promise as a carrier for nucleic acids, as it can selectively bind to angiogenic tumor blood vessels. In this way, anti-angiogenic therapy via cationic liposome-mediated systemic siRNA delivery could be achieved in cancer therapy. In the present study, we proved our assumption by preparing various kinds of polyethylene glycol (PEG)-coated siRNA/cationic liposome complexes (siRNA-lipoplexes) and screening the avidity of these siRNA-lipoplexes upon angiogenic tumor blood vessels by means of a murine dorsal air sac (DAS) model. The lipoplex, having a lipid composition of DC-6-14/POPC/CHOL/DOPE/mPEG$_{2000}$-DSPE = 20/30/30/20/5 (molar ratio) and a charge ratio of cationic liposome and siRNA = 3.81 (+/−), showed a higher binding index to newly formed blood vessels. Systemic injection with the lipoplex containing siRNA for the Argonaute2 gene (apoptosis-inducible siRNA) resulted in significant anti-tumor effect without severe side effects in mice with Lewis lung carcinoma. Our results indicate that the PEGylated cationic liposome-mediated systemic delivery of cytotoxic siRNA achieves anti-angiogenesis, resulting in the suppression of tumor growth.

1. Introduction

Small interfering RNA (siRNA) can degrade the complementary mRNA by RNA interference (RNAi) (Elbashir et al., 2001). Because the gene silencing effect of siRNA is potent and sequence-specific, siRNA has been applied not only as a powerful tool used to suppress targeted gene expression, but also as a promising therapeutic molecule against genetic diseases including cancer (Lares et al., 2011; Phalon et al., 2010).

Due to the issue of safety, to deliver siRNA into targeted cells, much more attention has been paid to non-viral systems instead of viral-vector systems (Akhtar and Benter, 2007). Cationic liposome is one of the most attractive non-viral systems, as it forms a complex with siRNA (siRNA-lipoplex) and enhances the cellular uptake of siRNA as a result of electrostatic interactions between lipoplexes (+) and the plasma membrane (−) of cells. Successful *in vitro* and *in vivo* gene silencing with the lipoplex has been reported (Chien et al., 2005; Spagnou et al., 2004). However, *in vivo* gene silencing by systemic injection with the lipoplex is still limited due to a rapid clearance of the lipoplexes from blood circulation and less accumulation of them into target tissues following intravenous injection (de Wolf et al., 2007).

Tumor angiogenesis is a formation of neovessels from pre-existing vessels in solid tumors, which is critical for the support of tumor growth and progression, not only by providing nutrients, oxygen, growth factors and other substances to tumor cells, but also by allowing metastatic cells into circulation (Folkman, 1971). Anti-angiogenic therapy is expected to be one of the most promising of cancer therapies. Intravenously injected cationic liposome can selectively bind to tumor angiogenic vessels (Campbell et al., 2002; Dass, 2003; Schiffelers et al., 2005; Thurston et al., 1998), although it is not yet clear which molecules on the endothelial cells of angiogenic vessels are related to the interaction of cationic liposome with the vessels. Cationic liposome is expected to be a promising carrier system for the delivery of siRNA to tumor-related angiogenic vessels. Successful delivery of siRNA, which can induce cellular death, to angiogenic vessels in solid tumors by cationic liposome may exhibit an anti-angiogenic effect, resulting in sufficient tumor growth inhibition.

To gain prolonged *in vivo* blood circulation properties, PEGylation is frequently performed on liposomes (surface modification of liposomes with PEG) (Allen and Cullis, 2004). But PEGylation increases the *in vivo* blood circulation time of siRNA-lipoplex.

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However, PEGylation hampers the gene silencing effect both *in vitro* and *in vivo* (Mishra et al., 2004; Remaut et al., 2007; Hatakeyama et al., 2011). This discrepancy concerning PEGylation in siRNA delivery is referred to as the PEG-dilemma (Hatakeyama et al., 2011). In the present study, we assumed that the PEG on the surface of siRNA-lipoplex would improve the blood circulation properties of the lipoplex, but it may attenuate the interaction of the lipoplex with angiogenic vessels in the solid tumor as well as promoting its uptake by endothelial cells. Therefore, optimization of the PEGylated siRNA-lipoplex formulation is necessary to overcome the barriers associated with the use of PEG and to consequently achieve successful siRNA delivery to angiogenic vessels and good therapeutic efficacy.

In the present study, therefore, we tried to design PEG-coated siRNA-lipoplex that can efficiently deliver siRNA to angiogenic tumor blood vessels. For this purpose, we prepared various PEG-coated siRNA-lipoplexes and tested their circulating properties and binding activity to angiogenic vessels by means of the murine dorsal air sac (DAS) model. The DAS model is an easy one to induce newly formed blood vessels under the skin of mice (Abu-Lila et al., 2009). Then, the tumor growth inhibition of selected PEG-coated lipoplex including apoptosis-inducible siRNA was assessed in mice bearing Lewis lung carcinomas.

2. Materials and methods

2.1. Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC); 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE); and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(methoxy[polyethylene glycol]-2000) ($mPEG_{2000}$-DSPE) were generously donated by NOF (Tokyo, Japan). A cationic lipid, *O*,*O*′-ditetradecanoyl-*N*-(α-trimethyl ammonio acetyl) diethanolamine chloride (DC-6-14) was purchased from Sogo Pharmaceutical (Tokyo, Japan). Cholesterol (CHOL) was purchased from Wako Pure Chemical (Osaka, Japan). DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate) and Lipofectamine 2000 (Lf2000) were purchased from Invitrogen (CA, USA). All other reagents were of analytical grade.

2.2. siRNA

siRNAs were chemically synthesized and purified through HPLC by Nippon EGT (Toyama, Japan). The sequences for siRNAs were as follows: siRNA for mouse Argonaute2 (siAgo2); sense, 5′-UGAGGCACUUACCAUCCAUTT-3′; antisense, 5′-AUGGAUGGUAAGUGCCUCATT-3′; non-silencing control siRNA (siNS), which sequence was targeted for GFP (Tagami et al., 2008); sense, 5′-GGC UAC GUC CAG GAG CGC ATT-3′; antisense, 5′-UGC GCU CCU GGA CGU AGC CTT-3′.

For the preparation of siRNA duplexes, the complementary antisense and sense strands in TE buffer (10 μM Tris–HCl, 1 μM EDTA (pH 8.0), DNase- and RNase-free grade (Nippon Gene, Tokyo, Japan)) were mixed in equal amounts, followed by heating at 90 °C for 1 min. The reaction mixture was then allowed to cool at room temperature. The quality of siRNA duplexes was checked using 15% PAGE. The final concentration of the duplex was set at 50 μM with TE buffer.

2.3. Preparation of cationic liposomes

The compositions of the cationic liposomes are listed in Table 1. Cationic liposomes were prepared as described previously (Tagami et al., 2009). Briefly, the lipids were dissolved in chloroform. After evaporation of the organic solvent, the resulting thin lipid film was hydrated with 9% sucrose to produce multilamellar vesicles (MLVs). The MLVs were sized by repeated extrusion through polycarbonate membrane filters (Nuclepore, CA, USA) with consecutive pore sizes of 400, 200 and 100 nm. The mean diameters and zeta potentials of the resultant liposomes were determined using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The liposomal phospholipid concentration was quantified using a Fiske and Subbarow phosphate assay (Bartlett, 1959). For the screening experiment, 1 mol% of the florescence lipid phase marker, DiI, was added to the lipid mixture before evaporation.

2.4. Preparation of PEG-coated siRNA-lipoplexes

The formulations of siRNA-lipoplexes were prepared as described previously (Barichello et al., 2011). siRNA solution (100–400 μg in 150 μl of 9% sucrose) and cationic liposome solution (40 mM in 150 μl of 9% sucrose) were mixed with charge ratios (+/−) from 1.91 to 7.62. The mixture was immediately vortexed (2500 rpm, Vortex-Genie 2, Scientific Industries, NY, USA) for 10 min at room temperature to form homogenous siRNA-lipoplexes. For PEGylation of siRNA-lipoplexes, a post-insertion technique was used as described previously (Ishida et al., 1999). Briefly, $mPEG_{2000}$-DSPE (5 mol% of total lipid) in 9% sucrose solution was added into siRNA-lipoplex solution, and the mixture was then gently shaken at 37 °C for 1 h. The incorporation efficiency of PEG-lipid into liposomes was more than 90% in HPLC analysis. To detect the free-siRNA in the prepared PEG-coated siRNA-lipoplex, electrophoresis was performed on 2% agarose gel in 40 mM Tris–acetate/1 mM EDTA buffer.

2.5. Animals and tumor cell lines

The murine hemangioendothelioma cell line EOMA was purchased from American Type Culture Collection (VA, USA). Both the highly metastatic pulmonary melanoma B16BL6 and the Lewis lung carcinoma (LLC) cell lines were purchased from Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University). The three cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Japan Bioserum, Hiroshima, Japan), 10 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (ICN Biomedical, OH, USA). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air incubator.

Male ddY mice and C57BL/6 mice, 5-weeks-old, were purchased from Japan SLC (Shizuoka, Japan). The experimental animals were allowed free access to water and chow, and were housed under controlled environmental conditions (constant temperature, humidity, and 12 h dark–light cycle). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the University of Tokushima.

2.6. Mouse dorsal air sac (DAS) model

The mouse DAS model was established as described previously (Abu-Lila et al., 2009). Briefly, a chamber was prepared by covering both sides of a Millipore ring (10 mm diameter, 3 mm thickness) with Millipore filters (0.45 μm pore size), and was then filled with a suspension of B16BL6 tumor cells (1×10^7 cells) in 0.18 ml of DMEM. The chamber was then implanted into the subcutaneous dorsal air sac created by subcutaneous injection of 10 ml of air in anesthetized male ddY mice. At day 6 after chamber implantation, different formulations of DiI-labeled PEG-coated siRNA-lipoplexes (25 mg lipid/kg mouse in 200 μl of 9% sucrose) were intravenously injected into the mice. At 8 h after the injection, the mice were euthanized, and the back skin attached to the chamber was removed.