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Combined delivery of the adiponectin gene and rosiglitazone using cationic lipid emulsions

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ABSTRACT

For the combined delivery of an insulin-sensitizing adipokine; *i.e.*, the *ADN* gene, and the potent PPARγ agonist rosiglitazone, cationic lipid emulsions were formulated using the cationic lipid DOTAP, helper lipid DOPE, castor oil, Tween 20 and Tween 80. The effect of drug loading on the physicochemical characteristics of the cationic emulsion/DNA complexes was investigated. Complex formation between the cationic emulsion and negatively charged plasmid DNA was confirmed and protection from DNase was observed. The *in vitro* transfection efficiency and cytotoxicity were evaluated in HepG2 cells. The particle sizes of the cationic emulsion/DNA complex were in the range 230–540 nm and those of the rosiglitazone-loaded cationic emulsion/DNA complex were in the range 220–340 nm. Gel retardation of the complexes was observed when the complexation weight ratios of the cationic lipid to plasmid DNA exceeded 4:1 for both the drug-free and rosiglitazone-loaded complexes. Both complexes stabilized plasmid DNA against DNase. The ADN expression level increased dose-dependently when cells were transfected with the cationic emulsion/DNA complexes. The rosiglitazone-loaded cationic emulsion/DNA complexes showed higher cellular uptake in HepG2 cells depending on the rosiglitazone loading, but not depending on the type of plasmid DNA type such as pVAX/ADN, pCAG/ADN, or pVAX. The drug-loaded cationic emulsion/plasmid DNA complexes were less cytotoxic than free rosiglitazone. Therefore, a cationic emulsion could potentially serve as a co-delivery system for rosiglitazone and the adiponectin gene.

1. Introduction

Adiponectin (ADN) is an adipocyte-produced protein hormone that circulates in the blood. ADN acts via the activation of AMP-activated protein kinase (AMPK) and decreased circulating ADN concentrations are associated with insulin resistance, obesity, and type 2 diabetes (Liu et al., 2008). Plasma ADN is reduced markedly in obese human and those with type 2 diabetes, suggesting that circulating ADN is related to the development of insulin resistance. ADN plays an important role in insulin sensitization in mammals (inhibiting gluconeogenesis and stimulating fatty acid oxidation) by activating AMP-activated protein kinase and peroxisome proliferator-activated receptor (PPAR) proteins in skeletal muscle, liver, and adipocytes (Astapova and Leff, 2012; Lustig et al., 2012).

Rosiglitazone is a member of the thiazolidinedione (TZD) class of agents used to treat type 2 diabetes. This class of agents improves glycemic control by improving insulin sensitivity. Rosiglitazone is a selective PPARγ agonist. The activation of PPARγ receptors regulates the transcription of insulin-responsive genes in the tissues in which they are found which include adipose tissue, skeletal muscle, and liver. PPARγ-responsive genes are involved in the control of glucose production, transport, and utilization, as well as in the regulation of fatty acid metabolism (Yamauchi et al., 2001). Additionally, TZDs have been shown to rapidly stimulate AMPK (Fryer et al., 2002; LeBrasseur et al., 2006) and may indirectly activate AMPK through the effects of PPARγ to stimulate ADN secretion (Suzuki and Eguchi, 2006; Kubota et al., 2006).

Therefore, both TZDs and ADN have been known to activate AMPK (Coughlan et al., 2014). Moreover, TZDs increase the production and plasma concentration of ADN. TZDs have weaker

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antidiabetic effects in ob/ob mice lacking the *ADN* gene than in ob/ob mice with it, and the activation of AMPK by TZDs is also attenuated in these mice, suggesting that ADN is required for the activation of AMPK by TZDs (Nawrocki et al., 2006). If rosiglitazone were available in combination with ADN, then the ADN might alleviate the side effect of rosiglitazone via dose control (Wiradharma et al., 2009). The combined delivery of a drug and gene is feasible because we previously combined drug-loaded lipid carriers and nucleic acids (Jeong et al., 2009, 2014; Davaa et al., 2010).

Cationic lipid emulsions have been prepared to overcome problems related to the solubility, encapsulation efficiency, and release of rosiglitazone (Davaa and Park, 2012). The encapsulation efficiency of a drug in a vehicle is of pharmaceutical importance, especially for optimizing efficacy and cost effectiveness. The cellular uptake of a drug is another factor to consider when maximizing its therapeutic efficacy. If a positive charge is introduced to the nanoparticles, the interaction between the positively charged vehicle and negatively charged cell surface would increase. Furthermore, cationic lipid emulsions have been used to transfer target genes into cells (Choi et al., 2004; Kang et al., 2009; Kim et al., 2014). Rosiglitazone would be loaded in the inner section of the emulsion, while plasmid DNA could interact electrically with the surface of a cationic emulsion. Therefore, the combination of rosiglitazone and the ADN gene is feasible for achieving synergistic or additive effects.

Here, we prepared a cationic emulsion that could efficiently co-deliver rosiglitazone and the ADN gene to a target cell with good physicochemical properties and high drug loading efficiency. The complexation ratios of the plasmid DNA and drug-loaded cationic emulsion were examined to optimize the co-delivery system. Then, the expression of ADN mRNA and cellular uptake of rosiglitazone using the optimized co-delivery system were compared.

2. Materials and methods

2.1. Materials

Rosiglitazone maleate was purchased from Masung & Co. (Seoul, Korea). 1,2-Dioleoyl-*sn*-glycero-3-trimethylammonium propane (DOTAP) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Polyoxyethylene sorbitan monooleate (Tween 80) was from Junsei Chemical (Japan). Castor oil, polyoxyethylene sorbitan monolaurate (Tween 20), potassium phosphate monobasic, sodium acetate, hydrochloric acid, chloroform, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and solubilization solution were purchased from Sigma–Aldrich (St. Louis, MO). Methanol and acetonitrile for high-performance liquid chromatography (HPLC) were obtained from SKYSOLTECH® from SK Chemicals (Seongnam, Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were purchased from Gibco® BRL (Grand Island, NY). Lipofectamine™ 2000 and TRIzol® were purchased from Invitrogen (Carlsbad, CA). Anti-adiponectin was from Cell Signaling Technology, Inc. (Beverly, MA, USA) and anti-β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-mouse and goat anti-rabbit antibodies were from Abfrontier (Seoul, Korea). All other chemicals were reagent grade and were used without further purification. The distilled and deionized water was used after sterilization.

2.2. Cell culture

Human hepatocellular carcinoma cells (HepG2) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 unit/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator supplied with 5% CO_2.

2.3. Purification of plasmid DNA

The plasmid DNA encoding adiponectin (pVAX/ADN and pCAG/ADN) (Nan et al., 2010; Davaa et al., 2013) was amplified in *Escherichia coli* DH5α and purified with a Plasmid Mega kit (QIAGEN, CA), according to the manufacturer's instructions. The integrity of the DNA preparation was confirmed on a 1% agarose gel.

2.4. Preparation of cationic lipid emulsions

The cationic lipid emulsion was prepared using the sonication method described by Choi et al. (2004). DOTAP and DOPE were used as the cationic and helper lipid, respectively. Rosiglitazone was dispersed in a mixture of castor oil and surfactants with constant stirring until the pre-emulsion concentrate became clear. An aliquot of rosiglitazone pre-emulsion concentrate was added to DOTAP and DOPE at a defined ratio. In brief, DOTAP, DOPE, castor oil, Tween 20, and Tween 80 were mixed at a weight ratio of 3:3:3:0.75:0.75 dissolved in chloroform (Davaa and Park, 2012). The organic solvent was removed on a rotary evaporator (RE-47 Yamato Scientific, Japan). The dried lipid film was flushed with nitrogen gas to remove traces of organic solvents and hydrated with 10 mL of phosphate-buffered saline (PBS, pH 7.4). Then, the hydrated lipid solution was vortexed and sonicated in a bath-type sonicator (Bransonic, Branson Ultrasonic, Danbury, CT) at 37 °C for 2 h. Finally, the prepared cationic emulsion was stored at 4 °C. To remove the unloaded rosiglitazone, lipids, and surfactants from the emulsion, the formulation was ultrafiltered through a 0.45-μm PVDF syringe filter (Leur Lock type; NSW Norm-Ject®, Whatman Inc.) (Davaa et al., 2010; Davaa and Park, 2012). The loading concentration of rosiglitazone in cationic emulsion was 662.3 ± 47.9 μg/mL.

2.5. Preparation of cationic lipid emulsion/DNA complexes

Drug-loaded or unloaded cationic lipid emulsions/DNA complexes were prepared by mixing plasmid DNA (pVAX/ADN or pCAG/ADN) and the cationic lipid formulation in various ratios in serum-free medium. The mixtures were incubated for 15 min at 37 °C to facilitate complex formation.

2.6. Measurement of droplet size and zeta potential

The droplet size distribution of the cationic emulsions and complexes was measured using a dynamic light-scattering spectrophotometer (ELS-8000, Otsuka Electronics, Japan) at a fixed angle of 90° at room temperature. The samples were diluted with deionized water, and then transferred into a quartz cuvette in the ELS-8000. The system was used in auto-measuring mode at 80 mV.

The zeta potential of the cationic emulsions and complexes was measured using an electrophoretic light-scattering spectrophotometer (ELS-8000, Otsuka Electronics) at an angle of ∼20° at 40 mV to assess the surface charge of vesicles after dilution with deionized water at room temperature. The data were analyzed using the ELS-8000 software supplied by the manufacturer.

2.7. Agarose gel retardation

Complex formation between the plasmid DNA and cationic emulsion was assessed using agarose gel electrophoresis. Various ratios (0.5–32, w/w) of lipid/DNA mixtures with a fixed amount of