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Hyaluronic acid-conjugated lipoplexes for targeted delivery of siRNA in a murine metastatic lung cancer model

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ABSTRACT

We have investigated the impact of hyaluronic acid (HA)-coating on the targeting capacity of siRNA lipoplexes to CD44-overexpressing tumor cells. Cellular uptake and localization of HA-lipoplexes were evaluated by flow cytometry and fluorescence microscopy and both methods showed that these lipoplexes were rapidly internalized and localized primarily within the cytoplasm. Inhibition of luciferase expression on the A549-luciferase lung cancer cell line was achieved *in vitro* using an anti-Luc siRNA. 81% of luciferase gene expression inhibition was obtained *in vitro* with HA-lipoplexes at +/− ratio 2. *In vivo*, in a murine A549 metastatic lung cancer model, the treatment with HA-lipoplexes carrying anti-luciferase siRNA led to a statistically significant decrease of luciferase expression as opposed to progressive increase with non-modified lipoplexes or NaCl 0.9%. The reduction of the expression of luciferase mRNA tumor of mice treated with HA-lipoplexes supported the inhibition effect due to siRNA. These results highlight the potential of HA-lipoplexes in CD44-targeting siRNA delivery.

1. Introduction

Downregulation of gene expression is a promising strategy that meets different applications in therapeutics. Small interfering RNA (siRNA) molecules present the inherent advantage of nucleic acid therapies consisting in the almost unrestricted choice of targets (Aagaard and Rossi, 2007). SiRNA has shown potentialities for the treatment of lung diseases including the treatment of inflammatory, immune and infectious diseases, cystic fibrosis (CF) and cancer (Nascimento et al., 2012). However, their clinical use, even for lung diseases, is still limited due to the same obstacles faced by other nucleotide-based therapeutics. Indeed, siRNA are rapidly degraded by nucleases showing half-lives in biological fluids of the order of seconds to minutes (Soutschek et al., 2004). Moreover, siRNA lacks selectivity for the targeted tissue (Aagaard and Rossi, 2007; Devi, 2006; Fattal and Barratt, 2009; Fattal and Bochot, 2008; Nascimento et al., 2012). Within the tissues, they do not cross cell membranes readily because of their negative charge, hydrophilicity and molecular size (Dykxhoorn and Lieberman, 2006; Fattal and Barratt, 2009). To overcome these limitations and enable siRNA delivery to their site of action, different nanocarriers systems have been investigated, including the biocompatible lipid-based liposomes, or lipoplexes. It was shown that surface modification of liposomes with high molecular weight hyaluronic acid (HA) can improve their efficacy by mediating active CD44 targeting in tumors (Arpicco et al., 2013b; Dalla Pozza et al., 2013; Glucksam-Galnoy et al., 2012; Landesman-Milo et al., 2013; Peer and Margalit, 2004a,b; Rivkin et al., 2010; Ruhela et al., 2014; Yang et al., 2013). HA is a glycosaminoglycan polymer composed of disaccharide units of *N*-acetylglucosamine and D-glucuronic acid linked together through alternating β-1,3 and β-1,4 glycosidic

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bonds. It is biocompatible, being the major component of the extracellular matrix. The native high molecular weight HA is non-toxic and non-immunogenic (Laurent and Fraser, 1992). It does not induce expression of genes involved in proliferation or inflammation (Noble, 2002) and counteracts proangiogenic effects of the HA oligomers (Deed et al., 1997; Dufay Wojcicki et al., 2012). At last but not least, HA can be utilized as an addressing molecule due to the expression of its membrane receptor, CD44, on tumor initiating cells (Al-Hajj et al., 2003) that are the main cells to target in order to avoid tumor relapse. Indeed, although some studies have focused on targeting CD44 using antibodies (Wang et al., 2012) or aptamers (Alshaer et al., 2015), most of them were related to the use of HA (Dosio et al., 2016). HA can also increase liposome circulation time due to possible dysopsonisation effects (Peer et al., 2003; Peer and Margalit, 2004b; Qhattal and Liu, 2011). Moreover, several groups have shown that it is possible to mediate siRNA delivery by nanotechnologies covered by HA such as polyethylenimine (Ganesh et al., 2013) or lipid particles (Landesman-Milo et al., 2013). For several years we have studied the effects of surface coverage of lipoplexes by HA through a HA-DOPE conjugate that is inserted within the lipoplex structure. The molecular organization of these lipoplexes was recently characterized (Dufay Wojcicki et al., 2012; Nascimento et al., 2015; Surace et al., 2009; Taetz et al., 2009). An improved and receptor-mediated transfection efficiency of breast and lung cancer cells overexpressing CD44 receptors was reported (Dufay Wojcicki et al., 2012; Surace et al., 2009). In a preliminary study, it was also shown that siRNA lipoplexes covered with HA could enter at a larger extent into A549 $CD44^+$ cells than Calu-3 $CD44^-$ cells (Taetz et al., 2009). However this last study did not demonstrate any inhibition effect provided by the siRNA. This is the reason why the aim of the present report was to provide evidences of success of such a strategy. HA-DOPE modified cationic siRNA lipoplexes were designed using a non-commercialized cationic lipid that has demonstrated promising transfection efficiency in different cell lines (Arpicco et al., 2004; De Rosa et al., 2008), the [2-(2-3didodecyloxypropyl)hydroxyethyl] ammonium bromide (DE). The effects of this modification on cell internalization were evaluated. Also, the ability of the lipoplexes to carry intact siRNA to the cytoplasm was assessed by testing gene expression inhibition on the A549-luc lung cancer cell line *in vitro* and for the first time in an *in vivo* lung cancer experimental model.

2. Materials and methods

2.1. Materials

The cationic lipid [2-(2-3didodecyloxypropyl)hydroxyethyl] ammonium bromide (DE) was synthesized as described (Arpicco et al., 2004). L-alpha-dioleylphosphatidylethanolamine (DOPE) and phosphatidylethanolamine conjugated to rhodamine (PE-rhodamine) were purchased from Avanti Polar Lipids distributed by Sigma Aldrich (Saint Quentin Fallavier, France). High molecular weight hyaluronic acid (HA) (sodium salt, 1600 kDa, purity of 95%) was provided by Acros organics (Geel, Belgium). pGL3 luciferase (firefly) and control siRNA (19 bp) were purchased from Eurogentec (Angers, France). The HA-DOPE conjugate was synthesized as described previously (Surace et al., 2009).

2.2. Liposomes and lipoplexes preparation and characterization

Liposomes of DOPE/DE at 1:1 w/w ratio were prepared in water by the ethanol injection method (Batzri and Korn, 1973; Taetz et al., 2009). The preparation protocol is described in the supplementary information. The HA-DOPE content of liposomes is expressed as mass ratio of HA-DOPE to other lipids (DE + DOPE) (10% refers to 1:10 w/w). Lipoplexes were prepared at charge ratios (+/− ratios) of 2 and 134 by adding one volume of the 3 mM liposome suspension into two volumes of siRNA solution at 11.05 or 0.16 μM, respectively, in an Eppendorf tube, and gently homogenizing by pipetting up and down. The ratios were calculated based on the fact that one positive charge is provided by 1 mol of DE and 38 negative charges are brought by 1 mol of siRNA. Suspensions of 15 μL −2.5 mL of lipoplexes were usually prepared and incubated for 1 h at room temperature before use. Lipoplexes diameter, zeta potential and stability in the presence of serum were characterized as described in the supplementary information.

2.3. Cell culture

A549-luc-C8 Bioware Cell Line, a luciferase-expressing cell line derived from A549 adenocarcinomic human alveolar epithelial cells, was purchased from Caliper Life Sciences (Hopkinton, USA). Cells were cultured using RPMI-1640 medium supplemented with 10% FBS, 50 units/mL penicillin and 50 units/mL streptomycin. They were maintained at 37 °C in a humidified atmosphere with 5% CO_2. To improve the homogeneity of luciferase expression and increase luminescence signals, a protocol for selection pressure was optimized. Before each experiment, cells were cultured for 12 days using the RPMI medium described previously with addition of 75 μg/ml Geneticin®G418 antibiotic (Gibco, Paisley, Scotland). CD44 expression on A549, A549-luc and G418-selected A549-luc cells was evaluated by flow cytometry (method described in the supplementary Information). Almost 100% of the cells expressed CD44 receptors on their surface, and the amount of receptors did not change after treatment with G418 (Table S1 and Fig. S1, Supplementary Information).

2.4. Cell viability

Cellular mitochondrial activity was evaluated after incubation with liposomes and lipoplexes using the 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyl tetrazolium bromide (MTT) test. Cells were seeded in 96-well plates at a density of 70,000 cells/mL and allowed to adhere. After 24 h, cells were rinsed with PBS and fresh serum-free medium was added to the wells. Liposomes and lipoplexes were diluted in RPMI serum-free medium and added to the wells at various lipid concentrations (0.3–272 μM). Six hours after incubation, serum was added to the wells at 10% v/v, and cells were incubated for 48 h. Then, 20 μL of a 5 mg/mL MTT solution was added to each well. After 2 h of incubation at 37 °C, the medium was discarded and 100 μL of DMSO was added to lyse the cells and solubilize the formazan crystals. The absorbance was measured with a micro-plate reader at 540 nm. Each liposome or lipoplex concentration was evaluated in triplicate, and the experiment was performed at least three times. Cell viability was expressed as the percentage of mitochondrial activity relative to the non-treated cells.

2.5. Lipoplexes uptake

Cells were seeded on 12-well plates at a density of 72,000 cells/mL and allowed to adhere. After 24 h, cells were rinsed with PBS, and fresh serum-free medium was added to the wells. Rhodamine-labeled liposomes and lipoplexes at +/− ratios 2 and 134 were diluted in RPMI serum-free medium and added into the wells at a final lipid concentration of 10 μM (67 nM of siRNA for lipoplexes at +/− ratio 2 and 1 nM of siRNA for lipoplexes 134), and incubated at 37 °C. After 2, 5, 24 or 48 h, supernatants were discarded, and cells rinsed twice with PBS and harvested by 1 × trypsine. This step was introduced to ensure that particles attached to the surface of cells, but not internalized, would not be taken into account in the flow cytometry measurements, possibly giving false-positive results.