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Tf-lipoplexes for neuronal siRNA delivery: A promising system to mediate gene silencing in the CNS

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ABSTRACT

Although RNAi-based gene silencing holds a great potential for treatment of neurological disorders, its application to the CNS has been restricted by low levels of tissue distribution and cellular uptake. In this work we report that cationic lipid-based vectors can enhance siRNA delivery to neurons both *in vitro* and *in vivo*. DOTAP:Chol liposomes associated with transferrin (Tf) and complexed with siRNAs (Tf-lipoplexes) were delivered to primary cultures of luciferase-expressing cortical neurons. Confocal microscopy studies revealed efficient cellular uptake of Cy3-labelled siRNAs after Tf-lipoplex delivery, which was reduced but not completely inhibited by blocking the Tf-receptor with excess Tf. Gene silencing was also evaluated after delivery of anti-luciferase or anti-c-Jun siRNAs. Our results demonstrate that Tf-lipoplexes achieve up to 50% luciferase and c-Jun knockdown, 48 h after transfection, without significant cytotoxicity. Similar results were observed *in vivo*, where a 40% reduction of luciferase activity was found in the striatum of luciferase mice. In addition, fluorescence microscopy studies showed extensive local distribution and internalization of Tf-lipoplex-associated Cy3-siRNAs without tissue toxicity. Overall, our results demonstrate that Tf-lipoplexes can mediate efficient gene silencing in neuronal cells, both *in vitro* an *in vivo*, which may prove useful in therapeutic approaches to neuronal protection and repair.

1. Introduction

The central nervous system (CNS) is the most complex and specialized organ in the human body, controlling almost all physiological processes through the action of neuronal cells. Due to their critical role, these postmitotic cells are irreplaceable and, once damaged, show little capacity for functional recovery. One of the major goals of current neuroscience research is to understand the mechanisms involved in neuronal degeneration and develop effective therapies to prevent neuronal cell death associated with neurological disorders.

Although numerous genes and toxic proteins thought to be involved in neuronal degeneration have been identified [1–3], the complexity of brain function and the crosstalk between the different cell death and survival pathways hampers the process of attributing a specific role to a particular protein. RNA interference (RNAi) has recently emerged as a powerful tool in functional genomic studies, allowing to dissect entire signalling pathways and understand the molecular mechanisms of neurobiological processes [4], thereby facilitating rapid identification and validation of possible therapeutic targets. Moreover, RNAi holds a pronounced therapeutic potential [5,6], since application of small interfering RNAs (siRNAs) may allow specific knockdown of selected toxic proteins, even when allele-specific silencing is needed, as in the case of dominantly inherited disorders [7,8].

Nevertheless, the development of RNAi-based therapeutics for *in vivo* application faces the same challenge common to all classes of drugs: achieving an efficient and sustained distribution into the target tissue at sufficient concentrations to accomplish a therapeutic effect [9]. The CNS presents additional obstacles for drug delivery, due to the presence of the blood-brain barrier and the necessity to target specific neuronal subpopulations. Since siRNA molecules need to gain access to the interior of the cell to promote mRNA cleavage, siRNA stabilization in the extracellular and intracellular environments during the delivery process and their efficient uptake by neuronal

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cells are major challenges to a successful therapeutic application [6]. Although siRNAs are more resistant to nuclease attack than single-stranded DNA and RNA, they are still prone to RNase degradation in biological fluids. Currently, chemically modified siRNAs with increased stability in the presence of serum are already commercially available and present an improvement for siRNA delivery *in vivo* [10–13]. Nevertheless, such modifications fail to solve both the poor pharmacokinetic properties common to all nucleic acid-based drugs and tissue specificity [14].

In this context, cationic liposomes can help to significantly increase the siRNA's circulation time and simultaneously reduce the dose required for efficient gene silencing by enhancing siRNA stability in serum and improving cellular uptake [15]. These lipid-based systems also present a high degree of versatility that allows surface modifications which result in a significant increase of their targeting capacity and transfection efficiency [15–19]. In this regard, DOTAP:Cholesterol/DNA complexes have been widely explored by us for both *in vitro* [15,17,20] and *in vivo* [16] gene therapy applications, mainly because of their capacity to mediate transfection in the presence of serum [18,21–23]. Also, transfection activity can be enhanced by the association of targeting ligands such as antibodies, peptides or proteins to the lipoplexes, which exploit the diversity of receptors existent at the surface of each cell type [19,24–27]. One useful cell-binding ligand is transferrin (Tf), an iron-transporting protein, which interacts with receptors ubiquitously expressed in various tissues [28]. Targeting transferrin receptors has been successfully applied for RNA and DNA delivery by coupling antibodies against these receptors [29,30], or transferrin to pegylated liposomes [31], or by associating the protein to cationic liposomes through electrostatic interactions [15,19,26,32]. In this regard, we and others have demonstrated that association of Tf to lipoplexes significantly enhances transfection efficiency by promoting lipoplex internalization in a large variety of cells [15–17,24,26,33–35], including neurons. In addition, we have reported that Tf can trigger cytoplasmic delivery of the carried nucleic acids through destabilization of the endosomal membrane under acidic conditions, thus further enhancing the transfection activity [20,24].

However, despite the widespread use of protein-associated cationic liposomes, there are still very few reports concerning their application for siRNA delivery to the CNS. We have recently reported the efficient siRNA complexation and gene silencing mediated by Tf-lipoplexes in glioblastoma and hepatocarcinoma cell lines [15]. In the present study, we applied the optimized Tf-lipoplexes to transfer siRNA targeting the firefly luciferase reporter gene into primary neuronal cultures obtained from transgenic NF-kB luciferase reporter mice. We also evaluated Tf-lipoplex-mediated siRNA delivery as well as luciferase and c-Jun silencing *in vivo*, after stereotactic injection in the striatum of the transgenic luciferase reporter mice. The results presented here demonstrate that Tf-lipoplexes can efficiently promote siRNA delivery to neuronal cells both, *in vitro* and *in vivo*, resulting in enhanced siRNA internalization and pronounced gene silencing effects, proposing a significant potential for therapeutic applications.

2. Materials and methods

2.1. Materials

The cationic lipid 1,2 dioleoyl-3(trimethylammonium)propane (DOTAP) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Iron saturated human transferrin (Tf) was obtained from Sigma (St. Louis, MO, USA). The Cy3-labeled non-specific siRNA sequence was purchased from Ambion (Austin, TX, USA). The anti-luciferase siRNA (luciferase Gl2 duplex) and the non-silencing siRNA used as control were obtained from Dharmacon (Lafayette, CO, USA). The anti-c-Jun siRNA (5'-AGTCATGAACCACGTTAAC-3') was obtained from Thermo Bioproducts. All other chemicals were obtained from Sigma unless stated otherwise.

2.2. Animals

All efforts were made to minimize suffering and the number of animals according to the guidelines of the German animal protection law and derived guidelines on the ethical use of animals. C57BL/6 mice were obtained from Charles River, Sulzfeld, Germany. NF-κB-luciferase-reporter mice (genetic background: C57BL/6) were used for the measurement of NF-κB transcriptional activity as described previously [36]. The NF-κB-luciferase transgene contained the firefly luciferase gene, driven by two NF-κB sites responding to p65/p50, p50/cRel and other dimer combinations of NF-κB as established in previous studies [37,38]. All animals were kept under controlled light and environmental conditions (12 h dark/light cycle, 23±1 °C, 55±5% relative humidity) and had free access to food (Altromin, Germany) and water. The NF-κB-luciferase reporter mouse strain was used because of several advantages of this transgenic mouse strain compared to other transgenic reporter mice. Luciferase protein expression can be easily quantified in various tissues including brain tissue as demonstrated previously [36,39]. The animals express luciferase in any cell under the control of a NF-kB binding promoter [38]. Therefore, there is a constitutive expression of luciferase in any tissue reflecting the constitutive activity of NF-kB under physiological conditions. It is not necessary to induce NF-kB or luciferase activity in these animals for the current application, since luciferase levels are low but easily detectable in all tissues without further manipulations.

2.3. Liposome and complex preparation

Cationic liposomes composed of DOTAP:Cholesterol (1:1 molar ratio) were prepared as previously described by Campbell [40] for *in vitro* application. Briefly, a mixture of 1 ml of DOTAP and 1.5 ml of cholesterol in chloroform (from stock solutions of 25 mg/ml DOTAP and 37.8 mg/ml cholesterol), was dried under nitrogen in order to obtain a thin lipid film. The film was dissolved in 100 µl of ultrapure ethanol and the resulting ethanol solution was injected into 900 µl of HBS buffer (Hepes-buffered saline solution, 20 mM Hepes, 100 mM NaCl, pH 7.4) maintained continuously under vortex, employing a 250 µl Hamilton syringe. The resulting MLV (multilamellar vesicles) were sonicated briefly to obtain SUV (small unilamellar vesicles) and diluted with HBS to a final lipid concentration of 1.43 mM (1 mg/ml). Alternatively, for *in vivo* application, the dried lipid film was hydrated in 1.6 ml of 5% HBG buffer (Hepes-glucose buffer: 5% glucose, 20 mM Hepes, pH 7.4) and sonicated for 5 min. The resulting liposomes were then extruded 21 times through two stacked polycarbonate membranes (50 nm pore diameter) and diluted in HBG buffer to a final DOTAP concentration of 22.5 mM. The liposomes were stored at 4 °C until use. The size of DOTAP:Cholesterol liposomes is approximately 120 nm, a single and homogeneous population being observed, and the zeta potential is +70 mV.

For the *in vitro* studies Tf-lipoplexes were prepared by pre-incubating a given volume of cationic liposomes (dependent on the desired (+/−) charge ratio) with iron-saturated human transferrin (32 µg/µg of siRNA) for 15 min, before mixing the necessary volume of siRNA stock solution to achieve a final siRNA concentration of 50 or 100 nM in each well. The mixture was further incubated for 30 min, at room temperature before delivery to cortical neurons in culture. Alternatively, for *in vivo* administration, Tf-lipoplexes prepared at a 2/1 lipid/siRNA (+/−) charge ratio were obtained by mixing 0.8 µl/animal of the liposome stock solution (22.5 mM DOTAP) with 0.5 µl/animal of human Tf solution (192 mg/ml in HBG), followed by 15 min incubation prior to the addition of 3 µg/animal of siRNAs. The resulting mixture was further incubated for 30 min. All formulations were used immediately after being prepared. When mixed according to this protocol, transferrin (which exhibits a net negative charge) binds electrostactically with the positive charges of the cationic liposomes, resulting in a slight decrease in the net positive charge of the formulation but allowing further