Journal of Controlled Release 153 (2011) 83–92

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Unconventional internalization mechanisms underlying functional delivery of antisense oligonucleotides via cationic lipoplexes and polyplexes

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ARTICLE INFO

Article history:
Received 20 January 2011
Accepted 27 April 2011
Available online 4 May 2011

Keywords:
Cellular delivery
Endocytosis
Internalization
Antisense oligonucleotides
Splice-shifting oligonucleotides
Intracellular trafficking
Cationic lipoplexes
Cationic polyplexes

ABSTRACT

There is mounting interest in developing antisense and siRNA oligonucleotides into therapeutic entities; however, this potential has been limited by poor access of oligonucleotides to their pharmacological targets within cells. Transfection reagents, such as cationic lipids and polymers, are commonly utilized to improve functional delivery of nucleic acids including oligonucleotides. Cellular entry of large plasmid DNA molecules with the assistance of these polycationic carriers is mediated by some form of endocytosis; however, the mechanism for delivery of small oligonucleotide molecules has not been well established. In this study, splice-shifting oligonucleotides have been formulated into cationic lipoplexes and polyplexes, and their internalization mechanisms have been examined by using pharmacological and genetic inhibitors of endocytosis. The results showed that intercellular distribution of the oligonucleotides to the nucleus governs their pharmacological response. A mechanistic study revealed that oligonucleotides delivered by lipoplexes enter the cells partially by membrane fusion and this mechanism accounts for the functional induction of the target gene. In contrast, polyplexes are internalized by unconventional endocytosis pathways that do not require dynamin or caveolin. These studies may help rationally design novel delivery systems with superior transfection efficiency but lower toxicity.

1. Introduction

There is a great potential for antisense and siRNA oligonucleotides to become mainstream therapeutic entities thanks to their high specificity and wide therapeutic target space as compared to small molecules. However, the pharmacological targets within cells are poorly accessible to oligonucleotides that are hydrophilic and often charged macromolecules [1,2]. In order to reach these intracellular targets, therapeutic oligonucleotides must cross numerous biological barriers following administration, of which transport across the plasma membrane and then trafficking to the intracellular site of action have been generally considered as the rate-limiting steps [1–3]. One strategy to overcome these biological barriers and allow successful delivery is to formulate oligonucleotides into nanoparticles with cationic lipids and polymers. This strategy has achieved significant success in cellular and animal studies [4–7] and generated promising results in Phase I clinical studies in which therapeutic siRNAs were delivered to tumors by a liposomal formulation [8]. Recently, polymer-based nanoparticles coated with transferrin were shown to deliver siRNAs to solid tumors in humans and cause gene specific RNAi activity [9]; this is the first siRNA clinical trial that used a polymer-based delivery system.

To design even superior delivery systems for therapeutic oligonucleotides, it is necessary to understand how the cationic carriers assist internalization and trafficking of the oligonucleotides. On the other hand, toxicity is often associated with these types of carriers [10], and this can only be avoided by understanding the mechanisms that cause it. Using pharmacological inhibitors of endocytosis, the internalization mechanisms of plasmid DNAs that are formulated with cationic lipids or polymers have been examined [11–13]. The results demonstrated that uptake of DOTAP/DNA lipoplexes is due to clathrin-mediated endocytosis, which is also responsible for the functional transfection [11,12]. On the other hand, PEI/DNA polyplexes enter the cells via a combination of clathrin-mediated endocytosis and the caveolar pathway; however, only the latter pathway leads to effective endosomal escape and thereafter functional transfection [11–13]. Another study further demonstrated that plasmid DNAs formulated with different cationic lipids may undergo different endocytotic pathways [14]. The plasmid DNA complexed with DMRIE-C follows the caveolar pathway, while that formulated with Lipofectamine LTX is internalized by clathrin-mediated endocytosis [14]. Genetic inhibition of dynamin, which mediates pinching off the vesicles from the plasma membrane, reduces cellular uptake of both DNA lipoplexes dramatically [14]. Interestingly, a recent

Abbreviations: SSO, splice-shifting oligonucleotide; eGFP, enhanced green fluorescence protein; RFP, red fluorescence protein; DN, dominant negative; L2K, Lipofectamine 2000®; PEI, Polyethyleneimine.

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doi:10.1016/j.jconrel.2011.04.029

study has revealed a different mechanism for cellular entry of siRNAs delivered by lipoplexes [15]. The majority of siRNAs that are formulated in lipoplexes entered the cells by endocytosis, however, this pathway fails to produce functional knockdown, whereas the minor portion of the siRNAs, which are internalized possibly via membrane fusion, generates the target gene suppression [15]. This study highlighted that oligonucleotides may utilize a cellular entry mechanism that is different from that for larger DNA molecules, even when they are delivered with the same carrier system.

In the current study we have examined internalization mechanisms of antisense oligonucleotides formulated into cationic lipoplexes and polyplexes using pharmacological and genetic inhibitors of endocytosis. A 'splice-shifting oligonucleotide (SSO)', which is designed to correct splicing of an aberrant intron inserted into an eGFP reporter gene, has been formulated with a cationic lipid Lipofectamine 2000® (L2K) or a cationic polymer jetPEI™ (PEI). Both are commonly used transfection reagents for plasmid DNA and oligonucleotides. Successful delivery of SSOs to the cell nucleus would lead to up-regulation of eGFP expression, providing a positive read-out for functional delivery. This study showed that the SSOs delivered by lipoplexes enter the cells largely by membrane fusion and this mechanism also account for the functional induction of the target gene. On the other hand, PEI/SSO polyplexes are internalized by unconventional endocytosis pathways that do not require dynamin or caveolin functions. Preliminary study also demonstrated the different temperature dependence of functional oligonucleotide delivery via lipoplexes and polyplexes.

2. Materials and methods

2.1. Synthesis and chemical characterization of SSOs

The SSO (5′-GTTATTCTTTAGAATGGTGC-3′) is 2′-*O*-Me oligonucleotide with phosphorothioate linkage. The SSO and its 3′-Tamra conjugate were prepared as previously reported [16]. In brief, oligonucleotides were synthesized using phosphoramidites of the ultraMILD-protected bases on CPG supports (Glen Research, Sterling, VA, USA) using a AB 3400 DNA synthesizer (Applied Biosystems, Foster City, CA, USA). After cleavage from the CPG support and deprotection, the oligonucleotides were purified by reverse-phase HPLC on a Varian HPLC system (ProStar/Dynamax, Walnut Creek, CA) and identified using MALDI-TOF mass spectroscopy on a Voyager Applied Biosystem instrument (Foster City, CA).

2.2. Cell lines, plasmids, and transfection

The Hela S3 cells, containing an aberrant intron inserted into the eGFP coding sequence, were a kind gift from Dr R. Kole (University of North Carolina) [17]. This stably transfected cell line was referred to as Hela/eGFP654 and was cultured in F12K medium (Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Sigma, St. Louis, MO) and 500 μg/ml Geneticin (Invitrogen). Human melanoma cells A375SM were cultured in DMEM (Invitrogen) plus 10% FBS. To construct A375SM cells expressing eGFP654, the eGFP654 cassette was cut from the plasmid pEGFP654 [17] at the *BamH I* and *Not I* site and inserted into pcDNA3.1(+)/hygro (Invitrogen) resulting in the plasmid pcDNA3.1/hygro/eGFP654. Stable transfectants were obtained by transfecting A375SM cells with pcDNA3.1/hygro/eGFP654 plasmid using an Amaxa Nucleoporation system as per manufacturer's instructions. Selection was carried out in culture media containing 200 μg/ml hygromycin B (Roche) for 2 weeks. Individual clones were picked and screened for luciferase induction by the SSO complexed with L2K. The single cell clone with the highest expression induced by SSO was referred to as A375/eGFP654 and used in further studies.

The plasmids encoding eGFP-dynamin dominant negative (DN), RFP-dynamin DN, and RFP-caveolin DN were kindly provided by Dr. JoAnn Trejo (University of California at San Diego, USA), Dr. Jennifer Lippincott-Schwartz (National Institutes of Health, USA), and Dr. David Marks (Mayo Clinic and Foundation, USA), respectively. Plasmids expressing mutant dynamin or caveolin were transfected into the Hela/eGFP654 cells using an Amaxa Nucleoporation system as per manufacturer's instructions. Briefly, one million Hela/eGFP654 cells were nucleofected using the Cell Line Nucleofector Kit V, program I-013, and 2 μg of plasmids. The following day cells were treated with the oligonucleotide formulated with transfection reagents, and uptake and functional induction of the oligonucleotide were analyzed by flow cytometry, whereas their intracellular distribution was observed by confocal microscopy.

2.3. Oligonucleotide transfection

Hela/eGFP654 or A375/eGFP654 cells were seeded on 24-well plates at 5×10^4 cells per well in various experiments. The following day, cells were treated with the SSO complexed with either a cationic lipid L2K (Invitrogen) or a cationic polymer PEI (Polyplus, Illkirch, France) as per manufacturers' instructions. The amounts of the transfection reagents and the oligonucleotide were optimized to achieve considerable transfection efficiency (>30%). To achieve optimal transfection with the SSO lipoplexes, 1 μl of L2K was diluted into 50 μl of Opti-MEM I (Invitrogen). After 5-minute incubation at room temperature, the diluted L2K solution was then mixed gently with an equal volume of Opti-MEM I containing 30 pmoles of the SSO followed by 20-minute incubation at room temperature. The lipid/SSO complex was then added to each well containing 500 μl Opti-MEM I. The resultant 0.6 ml dose solution containing 50 nM SSOs was incubated with cells for 4 h at 37 °C. The cells were subsequently washed with PBS, and then fresh culture media were added. Cells were cultured for another 24 hours before measuring the eGFP expression with flow cytometry.

The optimal transfection with the SSO polyplexes was achieved when the N/P ratio was 6.25. To prepare the polyplexes, 1 μl of PEI was diluted into 50 μl of 150 mM NaCl solution and was then mixed by vortex with an equal volume of NaCl solution containing 60 pmoles of the SSO. After 20-minute incubation at room temperature, the PEI/SSO polyplexes were then added to each well containing 500 μl Opti-MEM I medium, which made up the 0.6 ml dose solution containing 100 nM SSO. Following the 4-hour treatment at 37 °C, the cells were subsequently washed with PBS and then replenished with fresh media for another 24-hour culture. The functional delivery was examined by measuring the eGFP expression with flow cytometry.

Pharmacological inhibitors were used to identify possible endocytotic pathways and the following concentrations were selected based on the previous studies [16,18,19]: chlorpromazine, 25 μM; methyl-β-cyclodextrin (methyl-β-CD), 1 mM; amiloride, 100 μM; cytochalasin D, 2 μM. The cells were pretreated with the inhibitors for 30 min at 37 °C and then transfected with lipoplexes or polyplexes for 4 h at 37 °C in the presence of the inhibitors. The cells were washed with PBS and cultured in fresh media in the absence of the inhibitors for another 24 h prior to measuring eGFP expression with flow cytometry. In the experiment of temperature-dependent transfection, the cells were incubated at specified temperatures for 1 h and then transfected with the oligonucleotide formulations at the same temperatures for 4 h. The cells were washed, replenished with fresh media, and incubated for 24 h at 37 °C prior to functional assay. In the experiment of cellular uptake, the cells were treated for 2 h at 37 °C in the presence of the inhibitors, and then were trypsinized before evaluating the uptake with flow cytometry.

2.4. Particle size and zeta potential measurement

The average particle sizes of the lipoplexes in OPTI-MEM I and the polyplexes in 150 mM NaCl solution were determined using a Coulter N5 Plus Sub-Micron Particle Sizer (Beckman Coulter, Miami, FL, USA) at a fixed angle of 90° and a temperature of 25 °C. Light scattering intensity was maintained within the required range of the instrument (5×10^4