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Cellular uptake pathways of lipid-modified cationic polymers in gene delivery to primary cells

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ABSTRACT

Hydrophobic modifications have emerged as a promising approach to improve the efficiency of non-viral gene delivery vectors (GDV). Functional GDVs from non-toxic polymers have been created with this approach but the mechanism(s) behind lipid-mediated enhancement in transfection remains to be clarified. Using a linoleic acid-substituted 2 kDa polyethylenimine (PEI2LA), we aimed to define the cellular uptake pathways and intracellular trafficking of plasmid DNA in normal human foreskin fibroblast cells. Several pharmacological compounds were applied to selectively inhibit uptake by clathrin-mediated endocytosis (CME), caveolin-mediated endocytosis (CvME) and macropinocytosis. We found that PEI2LA complexes were taken up predominantly through CME, and to a lesser extent by CvME. In contrast, its precursor molecule, PEI2 complexes was internalized primarily by CvME and macropinocytosis. The commonly used 25 kDa PEI 25 complexes utilized all endocytic pathways, suggesting its efficiency is derived from a different set of transfection pathways than PEI2LA. We further applied several endosome disruptive agents and found that hypertonic media enhanced the transfection of PEI2LA by 6.5-fold. We infer that lipid substitution changes the normal uptake pathways significantly and transfection with hydrophobically modified GDVs may be further enhanced by incorporating endosome disruptive elements into vector design.

1. Introduction

Cellular delivery of exogenous DNA molecules to manipulate physiological functions at the genetic level has been an indispensable tool in both molecular biology studies and biotechnology applications. Clinical translation of gene delivery as a form of molecular therapy has been slow to progress largely due to the absence of gene delivery vectors (GDVs) that can satisfy both efficiency and safety requirements. Dis-armed recombinant viral vectors remain as the most efficient method of gene delivery at present. However, the risk of immunogenicity, residual infectivity and insertional mutagenesis currently precludes their wide spread use [1]. Ongoing efforts into the development of non-viral GDVs have yielded a large library of biocompatible materials capable with sufficient DNA packaging and delivery ability in pre-clinical models, but they have yet to achieve the efficacy that viral vectors are able to incite. Strategies to improve the efficiency and biocompatibility of cationic reagents for gene delivery typically involve grafting functional ligands such as peptides, lipids, sugars, or a combination thereof, to improve stability, targeting, uptake and sub-cellular trafficking capabilities of the vectors [2]. In that regard, hydrophobic modification of cationic reagents with lipid moieties was shown to improve membrane binding and enhance gene delivery efficiency [3]. Our group has demonstrated the feasibility of this approach by grafting several endogenous lipids to the low molecular weight (2 kDa) polyethylenimine (PEI2). The most effective polymer, namely linoleic acid substituted PEI2 (PEI2LA), displayed markedly enhanced transfection efficiency over its relatively ineffective precursor molecule in both cultured cell lines and tissue-derived primary cells [4,5]. We previously showed that the enhanced efficiency of PEI2LA was partly due to stronger

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association with the nuclear membrane, which was correlated with better nuclear uptake and subsequent transgene expression [5]. However, the specific uptake pathway employed as well as the subsequent intracellular trafficking events mediated by the PEI2LA remain to be elucidated.

Lipid substitution is expected to enhance gene delivery by promoting stronger binding of the polymer/DNA complexes to hydrophobic domains of the plasma membrane to increase uptake. Hydrophobic modifications can also alter the physicochemical properties of the complexes, leading to a change in the uptake pathways. Uptake pathways are vitally important in determining the efficiency of GDVs as it relates to the intracellular processing, trafficking and recycling of the internalized complexes. Uptake of assembled complexes are widely regarded to proceed via endocytosis [6,7]. Endocytosis is broadly defined into two categories, pinocytosis and phagocytosis, the latter of which is restricted to specialized cell types such as lymphocytes and macrophages. Pinocytosis is further sub-divided into clathrin-dependent endocytosis (CME), caveolin-mediated endocytosis (CvME), macropinocytosis, and clathrin-/caveolin-independent pathway. CvME was thought to be the uptake pathway most conducive to transfection owing to its non-acidic, non-degradative environment, which maintained the intracellular integrity of the nucleic acid cargo. CvME was shown to be the endocytic pathway leading to efficient transgene expression in COS-7 and HeLa cells for PEI-mediated transfection [8–10]. However, others suggested the CME as the most efficient uptake pathway, as it not only provided an acidic environment for PEI complexes to facilitate endosome disruption via the proton-sponge effect, but also facilitated movement of the transgene cargo proximal to the perinuclear region, which subsequently increased the propensity for nuclear import [11]. Yet, recent studies have also suggested that uptake via macropinocytosis is the most efficient route of entry leading to transfection in PEI25 [12]. Regardless of which endocytic pathway is the most effective route for transfection, the notion that a single pathway can be optimal for all GDVs may not be realistic. Transfection pathways vary among different cell types [13,14] and are likely to depend on the biochemical nature of the GDVs and the physicochemical properties of resulting complexes [15]. In that regard, mechanistic studies and further design optimizations for non-viral GDVs should be ideally investigated in clinically relevant primary cell lines so that clinical translation to both *ex vivo* and *in vivo* delivery settings can be streamlined. That was not the case for previous studies exploring the role of endocytic pathways on the efficiency of non-viral GDVs, where transformed cell lines were routinely used [8,9,13,14,16–18].

In this study, we employed normal human foreskin fibroblast (NHFF) cells to elucidate the mechanism of cell entry and trafficking of polymeric GDVs. NHFF cell is a versatile platform in this regard since it has clinical relevance in both cell-based therapy for the induction of pluripotent cells and in cutaneous gene therapy for skin regeneration and wound repair [19–22]. We aimed to identify the predominant endocytic pathway involved in the uptake of PEI2LA complexes in NHFF cells as compared to native PEIs (PEI2 and PEI25). We applied a series of pharmacological inhibitors that selectively inhibited CME, CvME and macropinocytosis to determine the uptake pathway by reduction. The specific activities of the inhibitors were defined by titrating the concentrations of the drug against cell viability and transfection efficiency. We further examined the intracellular distribution of the complexes and the role of endosome release as a rate-limiting step in the overall transfection. Conclusions derived from this study would not only provide mechanistic insight into the impact of lipid-moieties in GDV trafficking but would also have direct implication on the future design of polymeric GDVs for therapeutic gene delivery to NHFF cells.

2. Materials and methods

2.1. Materials

The 2 kDa branched PEI (PEI2; $M_n = 1.8$ kDa, $M_w = 2.0$ kDa), 25 kDa branched PEI (PEI25; $M_n = 10$ kDa; $M_w = 25$ kDa), Hanks' Balanced Salt Solution (HBSS, with phenol red), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), spectrophotometric-grade dimethylsulfoxide (DMSO), sucrose, chloroquine diphosphate salt, chlorpromazine hydrochloride, methyl-β-cyclodextrin (mβCD), genistein and trypsin/EDTA were obtained from SIGMA (St. Louis, MO). The PEI2LA was prepared according to the synthetic scheme outlined in [4], with LA:PEI2 feed ratio of 0.1, that gave a polymer with an average substitution of 1.2 linoleic acids per polymer. Opti-MEM® I Reduced Serum Media, Dulbecco's Modified Eagle Medium (DMEM; high and low glucose with L-glutamine), penicillin (10,000 U/ml), streptomycin (10 mg/ml) and non-essential amino acids (100×) were from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was from PAA Laboratories (Etobicoke, Ontario). The blank plasmid gWIZ (i.e., no functional gene product) and gWIZ-GFP (i.e., Green Fluorescent Protein mammalian expression plasmid) were purchased from Aldevron (Fargo, ND). The photosensitizer aluminium phthalocyanine disulfonate (AlPcS2a) was purchased from Frontier Scientific (Logan, UT, USA). Wortmannin and amiloride hydrochloride were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Hoechst 33258, Pentahydrate (bis-Benzimide) and Alexa Fluor® 488 labeled Dextran (10 kDa) were from Life Technologies (Burlington, ON).

2.2. Cell culture

NHFF cells were isolated from patients as described previously [23] and cultured in a basic growth medium comprised of DMEM containing 4.5 g/ml D-glucose, supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM l-glutamine, 0.1 mM MEM non-essential amino acids, 100 U/ml penicillin, and 100 μg/L of streptomycin. Cells were maintained in a humidified 37 °C incubator with 5% CO_2. NHFF cells passaged between 14 and 24 generations were used in this study, and were grown in multiwell plates for transfection studies. For both uptake and transfection studies, cells were seeded in 24-well plates at an initial seeding density of 3×10^5 cells/well.

2.3. Plasmid DNA (pDNA) labeling

The gWIZ-GFP plasmid is a 5757 bp mammalian expression plasmid, which contains a modified promoter from the human cytomegalovirus (CMV) immediate early (IE) genes. gWIZ-GFP was labeled with the fluorophore Cy3 using the Label IT® Tracker™ Intracellular Nucleic Acid Localization Kit (Mirus Bio, WI) as per manufacturer's instructions. Briefly, a 0.2 (v/w) ratio of dye to nucleic acid reaction mix was prepared and incubated at 37 °C for 90 min. Unbound free Cy3 molecules were removed by ethanol precipitation according to manufacture suggested protocol. Purified labeled pDNA was then suspended in ddH_2O. Labeling efficiency was determined by calculating the ratio of base to dye using the equation $(A_{base} * \varepsilon_{dye})/(A_{dye} * \varepsilon_{base})$ by measuring absorbance at 260 nm (base) and 550 nm (dye) using the values $\varepsilon_{Cy3} = 250{,}000$; $\varepsilon_{base} = 6600$ and $CF_{260} = 0.05$. The contribution of dye to the A_{260} reading was corrected by using the equation $A_{base} = A_{260} - (A_{dye} * CF_{260})$. Plasmid DNA labeled using the concentration outlined yielded approximately 300 Cy3 labels per pDNA.

2.4. Cytotoxicity assessment of endocytosis inhibitors by MTT assay

The cytotoxic effects of the uptake inhibitors were assessed using the MTT cell viability assay, in which the yellow tetrazolium salt (MTT) is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of DMSO. Briefly, NHFF cells were seeded in 48-well plates at a concentration of 1×10^5 cells/well. Once the cells reached a density of 50–60% or after 1–2 days, inhibitors were added and incubated in OPTI-MEM. After 4 h incubation, inhibitors were removed and cells were further incubated for an additional 20 h in fresh growth medium. To process cells for assay, MTT was added directly to the medium to a final concentration of 1 mg/ml, and incubated at 37 °C for 2 h; the supernatant was removed by inverting the plates to decant the liquid; crystals remaining at the bottom of the plate were dissolved in DMSO at 200 μl/well. The absorbance was measured at 570 nm using an ELx800 absorbance microplate reader (Bio-Tek, Winooski, VT). Cell viability was expressed as a percentage relative to untreated cells, which served as the control.

2.5. Preparation of complexes for transfection

Self-assembled polymer/pDNA complexes were formed by diluting pDNA and polymer solutions separately in equal volumes of salt-free buffer (20 mM HEPES, pH 7.4) for PEI25 and PEI2 or OPTI-MEM for PEI2LA. After 5 min of equilibration, the pDNA and polymer solutions were mixed together, vortexed for 5 s, and incubated at room temperature for 25 min. The volume of the complexes at this stage was 1/5 of the final transfection media volume (i.e., 100 μl complex volume in a total of 500 μl