Understanding internalization mechanisms of polyplexes

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Abstract: Gene therapy to the central nervous system has the potential to treat neurological diseases including amyotrophic lateral sclerosis, Huntington’s disease, and Parkinson’s disease. However, current nonviral gene delivery technology suffers from low in vivo transfection efficiency. It may be possible to increase the efficiency of gene transfer by directing vehicle internalization through specific pathways. In this work, we have investigated internalization of neuron-targeted and untargeted gene carriers by treating HeLa and neuron-like PC-12 cells with two drugs that inhibit the clathrin- and caveolin-mediated endocytic pathways. Transfection studies showed that transgene expression in PC-12 cells increased when clathrin-mediated pathways were inhibited and decreased when caveolin-mediated pathways were inhibited. In contrast, transgene expression in HeLa cells decreased when either clathrin- or caveolin-mediated pathways were inhibited. Inhibition of clathrin- and caveolin-dependent pathways affected targeted and untargeted materials similarly, indicating that targeting did not have a significant influence on internalization mechanism. This work suggests that the internalization pathway for efficient gene delivery is cell type-dependent.

1. INTRODUCTION

Gene therapy possesses the potential to treat a wide variety of medical conditions by remediating underlying genetic mutations. Using viral or nonviral carriers, nucleic acids can be introduced into cells to induce production of therapeutic proteins or to knock down expression of deleterious proteins. In recent years, nonviral approaches to gene delivery have been studied extensively as a safer alternative to traditional viral vectors, which may provoke an immune response or lead to insertional mutagenesis [5]. However, the transfection efficiency of nonviral vehicles in vivo must be improved to move toward human clinical application.

Targeting of drug and gene delivery vehicles to specific cell types is often achieved through the use of ligands, which affect cellular uptake mechanisms and in turn intracellular trafficking and processing [2]. Vehicles are internalized via two main endocytic pathways, clathrin-mediated endocytosis and caveolin-mediated endocytosis (Figure 1). Clathrin-mediated internalization occurs in a traditional endocytosis pathway involving endosomal acidification (pH 5-6) and trafficking to lysosomes (pH ~4.5). In contrast, endocytosis through caveolae, invaginations of the cell surface, proceeds when the cell membrane pinches off to form vesicles. Internalized vesicles then travel to pH-neutral caveosomes. Because a critical step in effective gene delivery is escape of the delivery vector from the endosome before its degradation as a lysosome, the neutral caveosomes of caveolar uptake may provide a more stable environment for internalized material as compared to the endosomal-lysosomal pathway. Alternatively, the relatively high efficiency of the gene delivery polymer polyethylenimine (PEI) is hypothesized to be related to its ability to act as a proton sponge, a mechanism that enables it to escape from endosomal vesicles [5]. Thus, some delivery vehicles may be dependent on intracellular trafficking to an acidifying endosomal pathway.

Nucleic acid delivery to the central nervous system may reduce symptoms of neurological diseases including amyotrophic lateral sclerosis, Huntington’s disease, and Parkinson’s disease, although an effective and specific delivery platform for targeting cells in the nervous system is still needed. Previous research has shown that Tet1, a peptide identified by phage display against the neuronal ganglioside GT1b [4], targets gene delivery vehicles to neuron-like cells. Vehicles modified with Tet1 have been shown to bind specifically to primary dorsal root ganglion cells, and to have higher transfection in differentiated PC-12 cells when compared to untargeted vehicles [3,6].
Figure 1. Gene delivery vehicle uptake proceeds via two main endocytic pathways with different intracellular trafficking mechanisms.

The increased transfection efficiency of such targeted nonviral gene delivery vehicles may depend on how they are internalized. Here, the specific internalization pathways utilized by Tet1-targeted and untargeted gene carriers are investigated in neuron-like PC-12 cells and HeLa cells using small molecule inhibitors. By determining the mechanisms for internalization of these vehicles and the effect mediated by Tet1 modification, we will achieve a greater understanding of the conditions and pathways leading to successful gene delivery.

2. MATERIALS AND METHODS

2.1 Cell culture

2.1.1 PC-12 cell culture

PC-12 cells were obtained from ATCC and were maintained in growth medium (F-12K medium supplemented with 15% horse serum, 2.5% fetal bovine serum, and 1% antibiotics-antimycotics) in a 37°C, 5% CO₂ environment. Medium was replaced every 2-3 days. Cells were passaged at 60-80% confluence by incubation with Trypsin-EDTA, followed by resuspension in 1 mL of growth medium and trituration through a fire-polished glass Pasteur pipette. For differentiation to a neuron-like phenotype, cells were plated on a poly-L-lysine coated flask in differentiation medium (F-12K medium supplemented with 1% horse serum, 100 ng/mL nerve growth factor [NGF], and 1% antibiotics-antimycotics).

2.1.2 HeLa cell culture

HeLa cells were obtained from ATCC and were maintained in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum and 1% antibiotics-antimycotics in a 37°C, 5% CO₂ environment. Medium was replaced every 2-3 days. Cells were passaged at 60-80% confluence by incubation with Trypsin-EDTA, followed by resuspension in growth medium.

2.2 Inhibitor cytotoxicity assay

Cytotoxicity assays were performed in triplicate. PC-12 cells were plated at 10,000 cells/well in 96-well plates and differentiated for 2 days with 100 ng/mL NGF. HeLa cells were plated at 5,000 cells/well in 96-well plates and allowed to adhere overnight. Cells were incubated for 2 h with chlorpromazine hydrochloride (Sigma Chemical Co., St. Louis, MO) at concentrations ranging from 0.78 to 100 μg/mL, genistein (Sigma, St. Louis, MO) at concentrations ranging from 1.95 to 500 μg/mL, or differentiation medium (PC-12) or growth medium (HeLa). Cells were then washed once
with phosphate-buffered saline, pH 7.4 (PBS) and incubated with fresh medium for an additional 46 h. To measure cell viability, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent for 4 h. Absorbance at 490 nm was read using a TECAN Safire2 microplate reader.

2.3 Tet1-PEI synthesis and characterization

The Tet1 peptide (HLNILSTLWKYRC) was synthesized and purified by HPLC by Peptron (Daejeon, South Korea). Tet1-PEI was synthesized as described previously [3,6]. Briefly, branched polyethyleneimine (PEI, MW 25 kDa; Sigma, St. Louis, MO) was modified with 2 mole equivalents of N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP; Pierce, Rockford, IL) in dimethylformamide for 4 h at room temperature. PEI was purified using a PD-10 column (GE Healthcare, Piscataway, NJ) and reacted with 2.4 mole equivalents of Tet1 peptide for 12 h. Tet1-PEI was purified using a PD-10 column. PEI concentration was determined using a copper (II) acetate assay as described previously [9]. Peptide conjugation was determined by absorbance readings at 280 nm using a UV/vis spectrophotometer.

2.4 Polyplex formulation

Polyplexes were formulated by adding an equal volume of polymer (PEI or Tet1-PEI) to nucleic acid at the desired ratio of polymer amine groups to nucleic acid phosphate groups (N/P ratio). The N/P ratio was calculated based on a PEI subunit of 43 g/mol and a DNA subunit of 330 g/mol. Polyplexes were incubated for 10 min at room temperature before use to allow for complete complexation.

2.5 Plasmid transfection

Transfections were performed in replicates of 6. PC-12 cells were plated at 25,000 cells/well in 24-well plates and differentiated for 2 days with 100 ng/mL NGF. HeLa cells were plated at 30,000 cells/well in 24-well plates and allowed to adhere overnight. Cells were washed with PBS and were incubated with 10 μg/mL chlorpromazine, 50 μg/mL genistein, or left untreated in 200 μL of OptiMEM for 1 h. Polyplexes at an N/P ratio of 5 were formulated as described above, using 1 μg of gWiz Luciferase plasmid DNA (Aldevron, Fargo, ND) in 20 μL for each sample, and added to the wells. Cells were incubated in a 37°C, 5% CO2 environment for 2 h. Cells were then washed once with PBS and incubated with fresh differentiation medium (PC-12) or growth medium (HeLa) in a 37°C, 5% CO2 environment for an additional 46 h. To collect lysate, cells were washed with PBS, lysed with 200 μL of 1x Reporter Lysis Buffer (Promega Corp., Madison, WI), and frozen at -80°C. Lysate was collected and centrifuged at 21,000 g at 4°C for 15 min. 20 μL of supernatant were assayed for luciferase expression using 100 μL of luciferin substrate (Promega Corp., Madison, WI). Luminescence was integrated for 1 s using a TECAN Safire2 microplate reader. Luciferase activity is reported in relative luminescence units normalized by protein content (RLU/mg), as measured by a BCA Protein Assay Kit (Pierce).

3. RESULTS

3.1 Inhibitor cytotoxicity

The drugs chlorpromazine and genistein have been used in the literature to block clathrin-mediated endocytosis and caveolin-mediated endocytosis, respectively [2,8]. Cell viability was tested following treatment with a range of inhibitor concentrations to ensure that the inhibitors did not cause cytotoxicity. MTS assays showed that PC-12 cells were viable in chlorpromazine concentrations up to 25 μg/mL and in genistein concentrations up to 62.5 μg/mL (Figure 2A). HeLa cells were viable in chlorpromazine concentrations up to 12.5 μg/mL and in genistein concentrations up to 125 μg/mL (Figure 2B).
Figure 2. 2-day differentiated PC-12 cells (A) and HeLa cells (B) were shown to be viable at low concentrations of the inhibitors chlorpromazine and genistein.

3.2 Transfection with selective pathway inhibition

2-day differentiated PC-12 cells and HeLa cells were pre-treated with 10 μg/mL chlorpromazine or 50 μg/mL genistein for 1 h prior to transfection with PEI or Tet1-PEI polyplex delivery vehicles. Inhibitors had limited effects on cell viability at the concentrations used in transfection studies. Transfection efficiency was evaluated using the luciferase reporter system. Differences in transgene expression between untreated and inhibited conditions were statistically significant (Figure 3). To compare the effects of inhibitors, luciferase activity was normalized to untreated cells. In 2-day differentiated PC-12 cells, luciferase activity showed a more than 60-fold decrease with genistein treatment and an increase with chlorpromazine treatment (Figure 4A). Transfection efficiency in HeLa cells decreased more than 10-fold with either chlorpromazine or genistein treatment (Figure 4B). There was no significant difference in expression with Tet1-modified vehicles as compared to untargeted vehicles.

Similar trends were observed for undifferentiated PC-12 cells as compared to the 2-day differentiated cells (data not shown).

4. DISCUSSION

In this work, PC-12 and HeLa cells were treated with inhibitors to selectively block two major routes for polyplex internalization. The drug chlorpromazine inhibits clathrin-mediated uptake by disassociating the clathrin-coated pits from the cell surface [10]. Genistein blocks caveolar uptake by inhibiting tyrosine kinases involved in caveolin-mediated endocytosis [7]. Transfection studies demonstrated that internalization pathways for successful gene transfer in PC-12 and HeLa cells are different. Transgene expression in 2-day differentiated PC-12 cells was only reduced when caveolar uptake was inhibited (Figure 4A). In fact, gene expression increased relative to untreated cells when clathrin-mediated pathways were inhibited; it is possible that inhibition of the clathrin-dependent pathways causes delivery vehicles to preferentially route through alternative pathways that ultimately lead to more efficient gene transfer. In contrast, gene expression in HeLa cells was greatly reduced when either clathrin- or caveolin-mediated pathways were inhibited (Figure 4B). This indicates that successful gene transfer occurs primarily when polyplexes are internalized via a caveolin-dependent pathway in PC-12 cells, whereas both pathways are important in HeLa cells.
Figure 3. gWiz Luciferase plasmid complexed with PEI and Tet1-PEI was delivered to 2-day differentiated PC-12 cells (A) and HeLa cells (B). Results are reported as the mean RLU/mg protein±SD for samples in replicates of 6.

Figure 4. Luciferase activity normalized to untreated cells.

In addition, inhibition of clathrin- and caveolin-mediated endocytic pathways affected targeted and untargeted materials similarly. It is likely that Tet1 targeting is not achieved via an influence on polyplex internalization mechanism but rather utilizes another specific receptor-ligand interaction that has not yet been characterized.

This work suggests that target cell type has a significant influence on internalization pathways of polyplexes. With a greater understanding of the internalization mechanisms used by specific cell lines, delivery vehicles can be tuned for optimal gene transfer.

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