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Intracellular trafficking pathways involved in the gene transfer of nano-structured calcium phosphate-DNA particles

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ABSTRACT

Nano-structured calcium phosphate (NanoCaP) particles have been proven to be a powerful means of non-viral gene delivery. In order to better understand the mechanisms through which NanoCaPs-mediated mammalian cell transfection is achieved, we have sought to define the intracellular trafficking pathways involved in the cellular uptake and intracellular processing of these particles. Previous work has indicated that NanoCaP-DNA complexes are most likely internalized via endocytosis, however the subsequent pathways involved have not been determined. Through the use of specific inhibitors, we show that endocytosis of NanoCaP particles is both clathrin- and caveolae-dependent, and suggest that the caveolaer mechanism is the major contributor. We demonstrate colocalization of NanoCaP-pDNA complexes with known markers of both clathrin-coated and caveolar vesicles. Furthermore, through the use of quantitative flow cytometry, we present the first work in which the percent internalization of CaP-DNA complexes into cells is quantified. The overall goal of this research is to foster the continued improvement of NanoCaP-based gene delivery strategies.

1. Introduction

Non-viral gene therapy remains an attractive alternative to viral approaches for the treatment of acute and chronic disease [1]. Non-viral carriers possess several advantages over viral approaches with respect to toxicity, immunogenicity and DNA carrying capacity, as well as tissue-specific targeting [2–4]. Several non-viral vectors are currently in development [1,5–16]. Among these, calcium phosphate (CaP) remain known for their tremendous potential as non-viral gene delivery vectors [17–22]. Previously, our group demonstrated the excellent potential of nano-structured calcium phosphate particles, or NanoCaPs, for use in non-viral gene delivery applications. Our system of NanoCaPs synthesis is novel and well-controlled, and results in consistently high transfection efficiency [23]. Multiple pathways have been identified for endocytosis, and differing mechanisms by which these pathways deliver transgenes are known to exist [24,25]. In this work, we seek to identify which endocytic mechanisms govern cellular uptake and subsequent intracellular processing of NanoCaPs. Our goal is the development of an even more effective CaP-based gene delivery agent, capable of overcoming the various extracellular and intracellular trafficking barriers associated with non-viral gene delivery [4,26].

Endocytosis is currently regarded to be the primary method by which non-viral gene delivery vectors are internalized [25]. There are multiple endocytic pathways including phagocytosis, clathrin-dependent endocytosis, caveolae-dependent endocytosis, macropinocytosis and clathrin-/caveolae-independent endocytosis. Recently, several authors have reported the significance of both clathrin- and caveolae-mediated pathways in lipid [27,28] and polymer [27,29–32] based gene delivery. However, although the results of several studies indicate endocytosis as the means of

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internalization for CaP-DNA complexes [20,33], the specific involvement of clathrin- and caveolae-mediated pathways in CaP-mediated gene delivery has never been assessed. Clathrin-mediated endocytosis, which is the most well-characterized uptake mechanism, is a multi-step process typically initiated by the strong binding of a ligand to a specific receptor on the cell surface. Receptor binding induces clustering and the eventual dissociation of clathrin-coated pits (approximately 100–150 nm) from the plasma membrane. Depolymerization of the clathrin-coats subsequently ensues and depending on the cargo, these uncoated vesicles (referred to as early endosomes, pH = 5.9–6.0), then either (1) mature to late endosomes (pH = 5.0–6.0) and ultimately to lysosomes, or (2) release their cargo, and are trafficked to their target organelle [25,34]. Caveolae are small (approximately 50–80 nm), uncoated invaginations of the plasma membrane rich in cholesterol and glycosphingolipids [35]. It is hypothesized that upon internalization, caveolae either fuse with larger, more complex, pre-existing membrane vesicles, deemed caveosomes, or are trafficked to early endosomes [36]. Macromolecules trafficked via the former route are not subject to acidification.

In this study, we assess the roles of clathrin- and caveolae-mediated endocytosis in both the cellular uptake as well as in the subsequent intracellular trafficking of our NanoCaPs-pDNA complexes. Using specific chemical inhibitors, we determine the involvement of these pathways in CaP-mediated gene delivery. Results of these studies are outlined in this manuscript.

2. Materials and methods

2.1. Materials

All chemicals, inhibitors, fluorophores, transfection reagents, and cell culture media used in these experiments were purchased as described below: ACS grade calcium chloride dihydrate (100.0%), biological grade sodium chloride (99.0%), ACS grade sodium phosphate tribasic dodecahydrate (98.0%), USP grade dextrose monohydrate (100.0%) and molecular biology grade HEPES free acid (100.0%) from Fisher Scientific (Pittsburgh, PA); SigmaUltra grade potassium chloride (99.0%), HPLC grade filipin III (>85%), phenylarsine oxide (PAO) (≥97%) and chlorpromazine hydrochloride (≥98%) from Sigma Aldrich (St. Louis, MO); pCMV luciferase (5 mg/ml) from Aldevron, LLC (Fargo, ND); *Label*IT® Fluorescein Labeling Kit from Mirus Bio (Madison, WI); Alexa Fluor 555 transferrin, Alexa Fluor 555 Cholera Toxin Subunit B (CTB) (recombinant), Dulbecco's minimum essential medium (DMEM) and penicillin/streptomycin (P/S) from Invitrogen (Carlsbad, CA); MTT cell growth assay kit from Chemicon (Temecula, CA) and characterized fetal bovine serum (FBS) from Hyclone (Logan, UT). The cell lines used in these experiments, specifically, the COS-7 African green monkey kidney fibroblast-like cells and the HeLa human epithelial cells, were both obtained from the American Type Culture Collection (ATCC) (Manassas, VA).

2.2. Synthesis of the NanoCaPs/pDNA complexes

Nanoparticles of CaP complexed with pDNA were synthesized as described previously [23]. Briefly, 125.0 μl of a calcium precursor solution, comprising 6.25 μl (1 μg/μl) of pDNA (pCMV luciferase), 15.53 μl of 2 M $CaCl_2$ and 103.22 μl of deionized water, was added to an equal volume of a HEPES Buffered Saline Solution (HBS), pH 7.5. A calcium-to-phosphate ion ratio (Ca/P) of 130 was used for all experiments. The calcium precursor was added to the phosphate precursor solution at an addition rate of 13.4 μl/s as the phosphate precursor was concurrently vortexed at 500 rpm.

2.3. Luciferase reporter gene expression

HeLa and COS-7 cells were cultured in 75 cm^2 flasks at 37 °C and 5% CO_2 in DMEM supplemented with 10% FBS and 1% P/S. All cells were maintained at sub-confluence and passaged every 2–3 days. A day prior to transfection, cells were seeded in 24-well plates at a concentration of 3.0×10^4cells/well. On the day of transfection, both HeLa and COS-7 cells were pre-treated with various concentrations of either filipin (concentrations ranged from 0.25 μg/ml–1.50 μg/ml) or phenylarsine oxide (PAO) (concentrations ranged from 0.025 μM to 0.200 μM), for either 1 h or 10 min, respectively, in fresh serum free medium. The NanoCaPs/pDNA complexes were then synthesized as described above and added to the wells (15.0 μl/well). Incubation was continued for an additional hour before the medium was replaced with fresh FBS-supplemented medium. Twenty-four hours later, these cells were harvested and analyzed for both luciferase expression, using a standard luciferase assay system (Promega, Madison, WI) as well as for total protein content using a standard bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Inc, Rockford, IL).

2.4. Cell viability assessment: MTT assay

The cytotoxic effects of PAO and filipin on both HeLa and COS-7 cells were assessed using a MTT cell viability colorimetric assay. Cells were seeded in 24-well plates at a concentration of 3.0×10^4cells/well. 24 h post-seeding, cells were treated with either filipin or PAO, for either 2 h or 1 h and 10 min, respectively, in fresh serum free medium. This incubation time represented the total amount of time the cells were treated with the inhibitors in the transfection experiment described above. Subsequently, the medium was replaced with fresh FBS-supplemented medium and the cells were incubated for an additional 22 h. Cell viability was then assessed using a MTT assay in the following manner. First, the medium was replaced with 250 μl of fresh serum medium per well and 25 μl per well of MTT dissolved in PBS. Second, the plates were incubated in a humidified incubator at 37 °C for 4 h. Third, 250 μl of an isopropanol/0.04 N HCl solution was added to each well. Fourth, the plates were incubated at room temperature (~25 °C) for 30 min under constant shaking. Finally, the absorbance was measured at both a test wavelength of 570 nm, and a reference wavelength of 690 nm, using a Perkin Elmer Victor 3V (Waltham, MA) plate reader. Cell viability was then expressed as percent cell viability relative to untreated cells, which served as the control.

2.5. Statistical analysis

The data were analyzed to test for mean viability and transfection efficiency differences among the concentration groups using a one way multivariate analysis of variance (MANOVA). The univariate post hoc tests were performed using the Student-Newman-Keuls procedure. Significance was considered to be less than $P = 0.05$.

2.6. Cellular uptake studies

Flow cytometry was used to assess the level of NanoCaPs/pDNA internalization in both HeLa and COS-7 cells. In this experiment, cells were seeded in 12-well plates at a density of 6.0×10^4cells/well, one day prior to transfection. 24 h post-seeding, the cells were pre-treated with either filipin (1.25 μg/ml used for both cells lines) or PAO (0.15 and 0.20 μM for HeLa and COS-7 cells, respectively) for either 1 h or 10 min, respectively, in fresh serum free medium. Following the pre-treatment, cells were transfected with the NanoCaPs/fluorescein-labeled-pDNA complexes, prepared as described above, for an additional hour (30.0 μl particles added/well). pCMV Luciferase was fluorescently-labeled with fluorescein (492 nm excitation and 518 nm emission) using a Mirus *Label*IT® Fluorescein Labeling Kit (Madison, WI). Subsequently, the serum free medium was removed. The cells were washed with serum free medium, harvested by treatment with trypsin/EDTA and were then re-suspended in ice cold phosphate buffered saline (PBS). The cells were then centrifuged and treated with trypan blue (TB) (0.04%) (MP Biomedicals, Irvine, CA). TB was used to quench the extracellular fluorescence of non-internalized particles [27,37]. The cells were subsequently washed twice with ice cold PBS, re-suspended in PBS and analyzed using a FACSort™ flow cytometer (Becton Dickinson, San Jose, CA, USA). Ten thousand gated, viable cells were collected for each sample [27,29]. All subsequent calculations were performed using WinMDI (Version 2.9) (Scripps Research Institute, La Jolla, CA, USA).

2.7. Confocal microscopy

Confocal microscopy was used to visualize colocalization between the internalized NanoCaPs/fluorescein-labeled-pDNA particles and the pathway markers Alexa Fluor 555 transferrin (Invitrogen, Carlsbad, CA) and Alexa Fluor 555 cholera toxin B (Invitrogen, Carlsbad, CA). For this, HeLa and COS-7 cells were seeded on glass Lab-Tek™ Chamber Slides (Nalge Nunc International, Rochester, NY, USA) at a cell density of 3.8×10^4cells per chamber (4 wells/chamber) a day prior to transfection. 24-h post-seeding, the cells were simultaneously treated in serum free medium with either Alexa Fluor 555 transferrin (6.4 μg/ml) or CTB (1.3 μg/ml) and the NanoCaPs/fluorescein-labeled-pCMV Luciferase complexes (28.2 μl per well) for 4 h. Subsequently, the cells were washed twice with serum-free medium and fixed with 3.7% paraformaldehyde. Gel/Mount (aqueous mounting medium with anti-fading agents) (Biomeda, Foster City, CA), was then added to reduce photobleaching effects. Each slide was then sealed with a coverglass (Fisher Scientific, Pittsburgh, PA). All slides were protected from light and stored at 4 °C until further analysis. Cells were then visualized using a confocal microscope (Olympus IX81). Laser 1, which was used for the NanoCaPs-fluorescein-labeled-pDNA complexes, had an excitation and emission wavelength of 488 and 520 nm, respectively. Laser 2, which was used for the endocytic trafficking markers, had an excitation and emission wavelength of 543 and 612 nm, respectively. Laser 3, which was used for differential interference contrast (DIC) microscopy, had an excitation wavelength of 543 nm. All images were taken using a 60× oil PLAPON objective lens (numerical aperture 1.42) and captured using Fluoview FV1000 software. Images were scanned using