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Superior transfection efficiency of phagocytic astrocytes by large chitosan/DNA nanoparticles

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ABSTRACT

Purpose: Mechanism study of why astrocytes isolated from experimental autoimmune encephalomyelitis (EAE)-induced B6 mice or after being exposed to inflammatory factors had the highest transfection efficiency to larger-sized, but not compacted, pspCS/pDNA particles.
Methods: Phosphorylatable short peptide conjugated chitosan (pspCS) was compounded with plasmid DNA (pDNA) at different N:P ratios to form pspCS/pDNA particles of different size and zeta potentials. These pspCS/pDNA particles were used for the transfection of astrocytes isolated from either EAE induced or healthy B6 mice. Transfection efficiency and cell permeability of the particles were determined by the internalization of radio [H3]-labeled plasmid and the expression of a luciferase reporter gene respectively. Phagocytosis of EAE-astrocytes was determined by the internalization of FITC labeled dextran beads. By comparing the transfection efficiency of differently-sized pspCS/pDNA particles to normal and phagocytic astrocytes, with or without cytochalasin D, a phagocytosis inhibitor, in the presence, the contribution of phagocytosis to cell permeability and transfection efficiency was evaluated.
Results: In vivo EAE-induction or *in vitro* inflammatory factors treatment transferred normal astrocytes to be phagocytic astrocytes which underwent phagocytosis, had the highest cell permeability and transfection efficiency to larger-sized pspCS/pDNA particles formed at lower N:P ratios. When phagocytosis was inhibited by cytochalasin D, both cell permeability and transfection efficiency of phagocytic astrocytes to larger were significantly decreased. Thereafter, particle size, not zeta potential, was verified as the key factor for determining whether the particles could be phagocytosed. In addition phagocytosis was successfully induced in ARPE-19 cells as well, which also improved the transfection efficiency of larger pspCS/pDNA particles.
Conclusion: A generally accepted concept is that the internalization of cationic polymer/pDNA particles, chitosan-DNA complex for instance, is mainly through the procedure of endocytosis of the transfected cells. More compacted particles with higher zeta potential were used to be considered had higher cell permeability and transfection efficiency. However, here we reported that phagocytosis is another important procedure for determining internalization and transfection efficiency of cationic polymer/pDNA nanoparticles, especially for advanced transfection efficiency of large pspCS/pDNA particles. Thus, for gene delivery applications, the environmental condition of the target cells should be seriously considered for selecting an appropriate gene transfer strategies.

1. Introduction

Several non-viral vectors have been successfully used for gene delivery, including lipofectamine 2000, L-alpha-dioleoyl phosphatidylethanolamine (DOPE), poly-L-lysine (PLL), polyethyleneimine (PEI), atelocollagen, and chitosan (CS) [1–4]. Among these, chitosan-derived vectors have gained increasing interest as a safer and more cost-effective delivery system for genetic material, including genomic DNA, plasmid DNA (pDNA), siRNA and oligonucleotides (ODN). We worked on improving the transfection efficiency of chitosan for a decade and reported that low molecular weight chitosan (5000–8000 Da) was a perfect carrier for transporting pDNA across cell membrane [5]. We had found that the main factor preventing the expression of loaded exogenes was an insufficient unpacking of pDNA from the chitosan

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carrier into a transcribable form. By means of increasing intracellular degradation of the chitosan carrier, alternating electrostatic interaction between chitosan carrier and loaded DNA or enhancing nuclear localization of CS/DNA particles, we successfully enhanced exogene expression to a large extend [6–8]. During these strategies, phosphorylatable short peptide (psp) conjugated low molecular weight chitosan (pspCS) was shown to effectively facilitate exogene expression and was successfully used as a gene delivery vector in our experimental in vivo application [9]. Currently we attempted to use pspCS as a gene delivery vector to transfect astrocytes for neural inflammation curing studies and found that astrocytes isolated from normal control and EAE-induced mice had significantly different responses to the transfection of pspCS/pDNA particles. Although compacted cationic polymer-pDNA particles with higher zeta potential are considered of having advance cell permeability and transfection efficiency, EAE-astrocytes were confirmed of being more sensitive to the transfection of larger pspCS/pDNA particles due to their preformation of phagocytosis which allowed more pDNA to be taken up by host cells.

2. Material and methods

2.1. Material

Female C57BL/6 (B6) mice (12–14 weeks old) were purchased from Shanghai experimental animal center of Chinese Academy of Sciences and maintained in the animal facilities of Tianjin Medical University. Institutional approval was obtained, and institutional guidelines regarding animal experimentation were followed. Chitosan(CS) was purchased from Sigma(Sigma-Aldrich Co. USA) in Molecular mass = 5000 Da (degrees of deacetylation approximate to 85%). Phosphorylatable short peptide conjugated chitosan (pspCS) was prepared by ourselves with previously described method [7]. Luciferase reporter gene plasmid pGL-3 basic and luciferase activity detection kit were purchased from Promega Com(Promega, USA). Linear polyethylenimine (PEI, MW 25 kDa) was obtained from Polysciences Inc. (PA, USA). Radio H3 labeled plasmid pGL-3 basic was prepared by ourselves. SM(PEG)4 (NHS-PEG4-maleimide) of LMW 513.50 and NHS-PEG-Malhex of HMW 5000 was obtained from Rapp Polymere (Tuebingen, Germany).

2.2. EAE induction of B6 mice

To induce EAE, C57BL/6 mice (n = 10) were immunized subcutaneously in 6 spots at the base of the tail and on the flank with 200 μl of an emulsion containing myelin oligodendrocyte glycoprotein (MOG_{35-55}) 150 μg. The peptide was emulsified in complete Freund's adjuvant (CFA) (Sigma, St. Louis, MO). Animals were evaluated every 1–2 days for clinical symptoms. Six mice, verified of having obvious EAE, were subjected to subsequent astrocytes isolation.

2.3. Astrocytes isolation and in vitro culture

Astrocytes were purified separately from the brains of normal and EAE-induced B6 mice (18 days post immunization). After mouse decapitation, brain structures were removed, and the meninges were carefully stripped off. Tissues from 6 mice of each group were pooled together, washed in phosphate-buffered saline (PBS) with 0.6% glucose, and cortical structures were dissociated into single cells using a neural tissue dissociation kit (Miltenyi Biotec Inc. CA, USA; #130-094-802), following manufacture's protocol. Dissociated cells were subjected to the purification of astrocytes using antibody-conjugated magnetic beads and automated MACs (Miltenyi Biotech, Germany). In brief, dissociated cells were firstly incubated with biotin conjugated anti-mouse GLAST (ACSA-1) antibody followed by an incubation with anti-biotin MicroBeads, and then collected microbeads labeled cells on an autoMACsTM separator column. Isolated astrocytes were cultured in DMEM/F12 supplemented with 10% FBS at 37 °C in a humidified 5% CO2, 95% air incubator. The medium was changed every second day until the cells reached confluence. For *in vitro* inflammatory cytokines or TLR ligand treatment, IFNγ (final concentration of 10 ng/ml), TNFα (50 ng/ml) or LPS (50 ng/ml) were added to astrocytes at 80% confluence. Two days after cells were thoroughly washed with DMEM/F12, and then subjected to the transfection and phagocytosis assay of pspCS/pDNA particles.

2.4. Preparation of pspCS/pDNA and PEI/pDNA nanoparticles

For the preparation of pspCS/DNA complex, plasmid DNA (pDNA) was dissolved in TE buffer to a final concentration of 0.2 mg/ml, and pspCS was dissolved in acetic buffer (pH 5.4) to a final concentration range of 0.1–2.0 mg/ml. Then, 1.0 μg plasmid DNA was mixed with an equal volume of pspCS solution of different concentrations to control the N:P ratio of pspCS:pDNA. Mixed solutions were vortexed instantly and incubated at room temperature for 30 min to allow complex formation. pspCS/pDNA complexes were subjected to agarose gel electrophoresis to verify complex formation by gel retardation. Linear PEI/pDNA nanoparticles were formulated in 5% glucose solution at various ratios of PEI nitrogen to pDNA phosphate (N/P ratio). Subsequently, 1 μg of pDNA was diluted, and linear PEI was transferred into the pDNA solution. The solution was vortexed for 20 s and left at room temperature for 30 min.

2.5. Particle size and zeta potential analysis

Nanoparticle size measurements were conducted using the Zetasizer Nano ZS (Malvern, Southborough, MA, USA). Briefly, the nanoparticles were suspended in deionized water at a concentration of 1 mg/ml. The size measurements were performed at 25 °C and at a 173° scattering angle. The mean hydrodynamic diameter was determined by cumulative analysis. The zeta potential determinations were based on the electrophoretic mobility of the nanoparticles in the aqueous medium and were performed using folded capillary cells in automatic mode.

2.6. Phagocytosis assay

Freshly isolated astrocytes from both healthy control and EAE-induced B6 mice were thoroughly washed with PBS, suspended in DMEM/F12 medium with 10% FBS, and seeded in 96-well plate at a density of 1×10^5/ml. Both control and EAE astrocytes were treated with phagocytosis inhibitor cytochalasin D [10] (final concentration, 10 μM; Sigma-Aldrich, St. Louis, MO, USA) or vehicle control for 30 min. A 100 μl solution of FITC-labeled opsonized dextran beads (OPDex), at a final concentration of 50 ng/ml, was added to each of the cells. After a 30-min intubation, cells were spun down and the supernatant was discarded. 50 μl of diluted trypan blue solution (5 mg/ml) was added to quench surface bound but not internalized fluorescence, with incubation at ambient temperature for 5 min. Cells were washed twice with PBS to remove any residual of trypan blue. Thereafter, cells were fixed with 4% paraformaldehyde, incubated for 15 min at room temperature and washed with PBS. Fixed cells were firstly stained with PE-labeled anti-GLAST antibody for 1 h at room temperature and then incubated with DAPI at 5 ng/ml for another 5 mins. Cells were washed twice and suspended in PBS, subjected to flow cytometric analysis and phagocytosis quantification. Flow cytometric analysis was performed in BD FACSCalibur. PE and FITC fluorescence were detected at FL2 and

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