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Intracellular gene delivery is dependent on the type of non-viral carrier and defined by the cell surface glycosaminoglycans

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ABSTRACT

Intracellular limiting steps and molecules involved in internalization and intracellular routing of non-viral gene delivery systems are still poorly understood. In this study, the intracellular kinetics of three different gene delivery systems calcium phosphate precipitates (CaP), polyethyleneimine (PEI) and N-[1-(2,3-dioleyl)propyl]-N,N,N-trimethylammonium chloride (DOTAP)) were quantified at cellular, nuclear, transcriptional and translational levels by using qRT-PCR. Additionally, a role of cell surface glycosaminoglycans (GAGs) was evaluated by performing the aforementioned studies in cells devoid of GAGs (pgsB-618) and cells lacking heparan sulphate (HS). The obtained data showed that the intracellular kinetics was dependent on the type of gene carrier and the weakest intracellular step varied between the carriers; rapid elimination of cell-associated pDNA in CaP, nuclear uptake in DOTAP and transcriptional and translational events in PEI mediated transfections. Overall, neither the amount of cell- nor nuclear associated pDNA correlated with transgene expression but the mRNA expression of the transgene correlated well with the expression at protein level. The nuclear uptake of pDNA in all cases was rapid and efficient thus indicating that the post-nuclear processes including transcription and translation steps have a critical role in defining the efficiency of non-viral gene delivery systems. Our study demonstrated that cell-surface GAGs are not essential for cell surface binding and internalization of gene delivery complexes, but they are able to define the intracellular routing of the complexes by leading them to pathways with high pDNA elimination.

1. Introduction

One of the main hurdles limiting the clinical application of the non-viral gene delivery systems is their poor *in vivo* efficiency. This is due to the multiple extra- and intracellular barriers that non-viral gene delivery systems need to overcome in order to trigger production of the therapeutic protein. One of these barriers is macromolecules called proteoglycans (PGs). They are composed of extremely negatively charged glycosaminoglycan (GAG) chains covalently attached to protein core, and are abundant both in the extracellular space and on the cell surface of all adherent cells [1]. Cell surface PGs are known to have a substantial role in cell physiology including cell growth, proliferation and migration [2,3]. They also act as receptors for example for growth factors, membrane penetrating peptides and viruses [4–6].

Interactions between non-viral gene delivery complexes and cell surface PGs are inevitable because of their opposite charges and because PGs encompass the entire cell surface. However, the effects of cell-surface PGs on non-viral gene transfer are controversial. Numerous studies have shown that cell surface PGs, especially heparan sulphate proteoglycans (HSPG), act as receptors for non-viral gene delivery systems [7–10]. However, our previous studies demonstrated that cell surface PGs have a dominant role in binding of non-viral gene delivery systems but the cellular uptake via cell surface PGs is not the most ""favourable route for efficient transfection [11–13]. Paris et al. [14] studied an involvement of one well-known cell surface HSPGs, syndecans, with the polyethyleneimine (PEI)-mediated gene delivery and showed that syndecan-1 slightly enhanced transgene expression whereas syndecan-2 dramatically inhibited it. Thus, the entire role of cell surface PGs in non-viral gene transfer is not defined as yet and no studies on influence of cell surface PGs on pre- and post-nuclear kinetics of non-viral gene delivery complexes exist to date.

After cell surface binding via electrostatic interactions, non-viral gene delivery complexes are internalized by endocytosis [15,16]. After internalization, the complexes are trapped inside the endolysosomal compartments and are enzymatically degraded unless they manage to escape and/or release their cargo to the cytoplasm. Many groups have clarified the intracellular processing of the non-viral carrier mediated plasmid DNA (pDNA) toward the nucleus [15,17–27]. Several studies have shown that the amount of pDNA taken up by the cells does not correlate with nuclear uptake [11,17,22], and neither the cellular nor the

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nuclear uptake efficiencies correlate with transgene expression at protein level [23–25]. However, the impact of nuclear and post-nuclear kinetics, especially at the transcriptional level, in the overall gene delivery efficiency is still poorly understood, although the importance of these steps has recently been emphasized [23–27].

In this study, we clarified the role of cell surface GAGs on the intracellular kinetics of various well known non-viral gene delivery complexes composed of pDNA and cationic carrier PEI (polyplex) or DOTAP (lipoplex), or calcium phosphate precipitates (CaP). The studies were performed with parent CHO cell line and two CHO mutant cell lines, pgsB-618 and pgsD-677. PgsB-618 cell line is totally lacking cell-surface GAGs, whereas pgsD-677 are devoid of cell-surface HS but expressing total amount of other cell-surface GAGs (CS and HA) at higher level than parent CHO. Intracellular kinetics of aforementioned gene delivery systems were quantified at cellular, nuclear, transcriptional and translational levels as a function of time by using qRT-PCR.

2. Materials and methods

2.1. Cell culture

CHO (Chinese hamster ovary) cells, a kind gift from Dr. Seppo Ylä-Herttuala (University of Eastern Finland, Finland), were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (*v*/*v*) foetal bovine serum (FBS; Gibco), 2 mM L-glutamine (EuroClone) and 100 units/ml penicillin–streptomycin (PEST; EuroClone). The mutant cell lines pgsB-618 and pgsD-677 were purchased from the American Type Culture Collection (Manassas, VA, USA). PgsB-618 cells are devoid of galactosyltransferase I activity and thus are not able to produce any proteoglycans. These cells were grown in a medium containing Ham's F12 (Gibco), 10% (*v*/*v*) FBS, 2 mM L-glutamine and 100 units/ml PEST. PgsD-677 cells are lacking heparan sulphate polymerase activity which is required for heparan sulphate synthesis. This cell line was maintained in F12K Nutrient Mixture, Kaighn's (Gibco), complemented with 10% FBS and 100 units/ml PEST. All cell lines were grown at 37 °C in 7% CO_2 and were subcultured 2–3 times a week.

2.2. Plasmid DNA (pDNA)

The CMV-driven luciferase reporter plasmid (pCLuc4) was donated by Dr. F. C. Szoka, Jr. (UCSF, San Francisco, USA). The plasmids were amplified in *E. coli* and purified in ion-exchange column (Qiagen, Hilden, Germany). The purity and the concentration of the plasmid were determined by absorbances at 260 nm and 280 nm.

2.3. Cationic polymers and lipids

PEI with an average molecular weight of 25 kDa was obtained from Sigma-Aldrich Co. and was used as a 10 mM aqueous stock solution [28].

Cationic lipid N-[1-(2,3-dioleyl)propyl]-N,N,N-trimethylammonium chloride (DOTAP) was purchased from Avanti Polar Lipids Inc. DOTAP liposomes were prepared by evaporating chloroform from the stock solution (10 mg/ml) of lipids under vacuum. A completely dried thin film layer of lipids was rehydrated in sterile water to final concentration of 3.2 mM and sonicated until translucent solution was formed. Liposomes were stored at +4 °C in argon.

2.4. Preparation of gene delivery systems

Polyplexes and lipoplexes—Gene delivery complexes consisting of pDNA and PEI or DOTAP were prepared at an optimal transfection N/P ratio of +/− 4 by adding pDNA in water (2 μg/75 μl) to an equal volume (75 μl) of carrier. The complexes were mixed gently and incubated for 20 min at room temperature before adding to the cells.

Calcium phosphate precipitates (CaP) were prepared according to the method described by O'Mahoney and Adams [29]. Briefly, pDNA (2 μg) diluted in water (20 μl) was strongly mixed with 0.5 M $CaCl_2$ solution (20 μl). After 10 min incubation at room temperature, a buffer composed of 0.28 M NaCl, 1.5 mM Na-phosphate, 50 mM BES, pH 7 (40 μl) was added rapidly to the first solution and mixed thoroughly. The solution was incubated for 15 min at room temperature before adding to the cells.

2.5. Transfection procedures

The cells were seeded on six-well plates at a density of 4×10^5/well (CHO) or 3×10^5/well (pgsB-618 and pgsD-677), except for nuclei isolation studies in which the cells were seeded in 10 cm Petri dishes at a density of 1.5×10^6/dish (CHO) or 1×10^6/dish (pgsB-618 and pgsD-677). After 24 h, fresh and serum-free medium was changed and the complexes (2 μg of pDNA/well or 10 μg of pDNA/Petri dish) were added to the cells. After 4-hour exposure the complexes were removed, the cells were washed twice with 1×PBS and a fresh growth medium was added. The samples for determination of cell associated pDNA and luciferase expression, both at mRNA and protein levels, were taken at the time of complex removal (=4 h time point) followed by sample collection every 12 h up to 5 days. Additionally, in cell association studies, after CaP-mediated transfection, the samples were also taken during the first 12 h every half an hour. In nuclear associated studies, the samples were collected before and after complex exposure followed by sampling at 8, 12, 24, 48, 72, 96 and 120 h time points.

2.6. Determination of cell-associated pDNA

The amount of cell-associated pDNA was determined from the whole cell samples described earlier [20]. At specific time points (see chapter 2.5), the cells were lysed with sterile water (500 μl/well) after PBS washes and stored at −70 °C prior to analysis. After rapid thawing, the samples were diluted 1:200 in water and were treated with 9 μM heparan sulphate (Sigma) for 15 min at room temperature in order to release pDNA or to relax the complexes. The samples were further diluted 1:10 in water and the amount of pDNA was quantified by qRT-PCR.

2.7. Nuclei isolation

Cell nuclei were isolated and purified by using iodixanol method introduced by Cohen et al. [23] with minor modifications [24,25]. Briefly, at certain time points (see chapter 2.5), the cells were washed twice with PBS, detached with trypsin-EDTA (Invitrogen) and centrifuged. The cell pellet was further washed with PBS, centrifuged and resuspended in PBS. After repeating this step two more times, the final cell pellet was gently resuspended in 500 μl of hypo-osmotic homogenization medium (1 mM KCl, 10 mM $MgCl_2$, 5 mM Hepes-NaOH, pH 7.4). After 25 min incubation on ice, the cell membranes were disrupted by passing the cells 15 times through 25G needle using 1 ml syringe. This step was optimized for each cell line by visualizing Trypan blue-stained nuclei under microscope and by measuring the release of lactate dehydrogenase (LDH) enzyme from the cells by using LDH assay kit (Promega Co., USA). After cell membrane disruption, the samples were centrifuged (4000 rpm, 5 min, 4 °C) and the pellet was resuspended in 500 μl of 25% OptiPrep™ solution (Iodixanol 60% *w*/*v*, Sigma) in homogenization buffer (25 mM KCl, 5 mM $MgCl_2$, 20 mM Tricine-KOH, pH 7.8). A discontinuous iodixanol gradient was prepared in 2 ml Eppendorf tube. First, 30% iodixanol (500 μl) was overlaid on the 35% iodixanol (500 μl). After this the 25% iodixanol solution containing cell samples was layered gently on the top of the gradient. The gradient was centrifuged (12 000 rpm, 20 min, 4 °C) and the purified cell nuclei fraction was collected from the 30/35% boundary layer. The amount of nuclei was counted by a hemocytometer (Bűrker) after staining with 0.5% Trypan blue in PBS. The samples were stored at −20 °C prior to the analysis. After thawing the nuclei samples, the nuclear membrane was disrupted with 0.005% (*w*/*v*)

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