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Live imaging of transgene expression in Cloudman S91 melanoma cells after polyplex-mediated gene delivery

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ABSTRACT

Utilizing nanoparticles made of cationic polymers as gene carriers is a promising approach in cancer gene therapy. One of the major requirements for successful gene delivery is DNA translocation into the nuclei of cancer cells. Nuclear envelope breakdown during mitosis has been considered as the most favorable opportunity for DNA translocation to the nucleus. Here, we aimed to study the influence of mitosis on polyplex-mediated gene delivery using time-lapse microscopy as a safe and accurate tool. Studying of reporter gene expression on a single cell level enabled to confirm the significance of cell division for gene delivery to Cloudman S91 melanoma cells, in spite of minor mitosis-independent transfection, and to discover some important details of polyplex delivery process. We have found that cell division can result in only one post-mitotic transfected cell of the two that could indicate non-uniform distribution of a very small number of intact plasmid DNA between daughter cells. According to our data, the shorter the time interval from polyplex addition to cell division, the longer time is required for the start of reporter gene expression after completed cytokinesis that presumably is a result of gradual polyplex dissociation in cell. Most probably, the development of new gene delivery carriers which would combine the strong ability to protect DNA and ability to release it during mitosis can provide an increase in intact DNA molecule number per cell, uniform DNA distribution between two post-mitotic cells, and fast reporter gene expression resulting in superior transfection of proliferating cells.

1. Introduction

The ideal gene delivery systems must be safe and highly effective in terms of transfection and specificity [1]. Cationic polymer-based nanoparticles have a high potential in the field of cancer gene therapy because of the lack of immunogenicity and simplicity of production as compared, for example, with viral vectors [2]. The development of new approaches to enhance gene transfer efficacy is possible only due to deep insight into the mechanisms involved in intracellular trafficking of gene delivery vectors. Intracellular transport of cationic polymer/DNA-based nanoparticles includes binding to the cell surface, endocytosis, endosomal escape, moving through the cytosol and translocation to the cell nucleus [3]. Passing through each step may be accompanied with DNA release from the complex [4,5]. Although endosomal escape is considered as a major bottleneck of successful polyplex-mediated gene delivery [6,7], DNA translocation into the nuclear interior seems to be another critical and poorly understood step of polyplex intracellular transport. It should be noted that nuclear import of macromolecules, primarily proteins, occurs in a size-dependent manner: molecules less than 9 nm (or 40 kDa in mass) are able to diffuse through the channel of nuclear pore complex (NPC) with rates depending on size, whereas import of larger molecules is an energy- and signal-dependent active process [8,9]. Regarding DNA transfer, microinjection experiments showed that 200–300 bp is the upper limit size of linear DNA, which can diffuse through NPC relatively freely [10]. At the same time, exploiting NPC-mediated active transport due to covalent or non-covalent electrostatic binding of oligopeptide nuclear localization sequence to DNA in order to facilitate nuclear targeting has had the limited success [11]. Perhaps, the low efficiency of this approach is due to combination of DNA instability in cytosol and its restricted mobility in cytoplasm [12,13] primarily due to thick network of actin filaments that hampers DNA transfer toward the nucleus [14]. It has been generally accepted that the most probable way of polyplex-delivered DNA entry is a passive translocation during mitosis while the nuclear envelope is dismantled [15]. The evidence for supporting this hypothesis is based on two observations. Firstly, cytoplasmic microinjection of plasmid DNA causes superior transfection efficiency of cells which had divided as compared with undivided ones [16]. Secondly, there are some indirect data about

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enhancement of gene expression after transfection with polyplexes in proliferating cells in contrast to non-dividing ones obtained using several crude techniques namely analysis of gene expression after cell synchronization, elutriation [17–19] or staining of cells with lipophilic membrane dye [20].

Here, we aimed to study in details the influence of mitosis on polyplex-mediated gene delivery, DNA distribution between two daughter cells and subsequent gene expression. It should be noted that the abovementioned methods, applied for investigation of the relationships between cell division and gene delivery, provide only averaged data on a whole cell population in spite of excellent statistics. Furthermore, the use of lipophilic dye as well as cell elutriation and synchronization, which suppose mechanical, low temperature or chemical impacts, may potentially promote stress in cultured cells affecting key cellular functions including endocytosis, cytoskeleton assembly and cell cycle progression. For these reasons, we used intravital time-lapse microscopy as a safe and more accurate technique. It is very important to emphasize that the applied method enables to carry out analysis of reporter gene expression in individual cells and completely excludes perturbation of cellular processes that may non-specifically impact intracellular transport of polyplexes and transfection. Additionally, this method also makes it possible to investigate the fate of fluorescently labeled DNA in transfected cells.

As a model gene delivery system, we used the previously described [21] polyethylenimine–polyethylene glycol–MC1SP block-copolymer (PEI–PEG–MC1SP) with MC1SP-peptide containing both nuclear localization sequence (NLS) and a ligand moiety, specific for melanocortin receptor-1 (MC1R) overexpressed on melanoma cells [22–24]. Obtained results determined the significant role of mitosis for polyplex-mediated gene delivery, although in a minor subpopulation of transfected cells transgene expression occurs without prior mitosis. Moreover, our data enable to elucidate some details of DNA translocation to the cell nuclei and following gene expression which can be useful in the development of advanced gene carriers.

2. Materials and methods

2.1. Polymeric nanoparticles

Block-copolymers of linear 25 kDa polyethylenimine (PEI) (Polysciences, Warrington, PA), and heterobifunctional polyethylene glycol MAL-dPEG24™-NHS ester (PEG) (Quanta BioDesign, Powell, OH), were synthesized as described previously [4,21]. On the next step, MC1SP-oligopeptide (NLS^+) CGYG*PKKKRKV*SGSG**SSIISHFRWGKPV,** or (NLS^-) CGYG*PK**T**KRKV*SGSG**SSIISHFRWGKPV** (Rusbiolink, Moscow, Russia), were covalently attached to the PEI–PEG block-copolymer [21]. Replacement of ^{7}Lys by ^{7}Thr completely abolishes active nuclear translocation of macromolecules with such a motif across NPC [25]. The PEI concentration of the conjugate was measured by a copper assay [26] at 620 nm. The amount of grafted PEG was quantified by a method described by Gong et al. [27]. The MC1SP concentration was determined by the increase in amino group concentration [28].

Plasmids pCMV-NIS (kindly provided by Alexei Kuzmich, Laboratory of human genes structure and functions, Institute of Bioorganic Chemistry of the RAS, Moscow, Russia) and pTurboYFP (Evrogen, Moscow, Russia), encoding sodium-iodide symporter (NIS) and enhanced yellow fluorescence protein (YFP), respectively, under cytomegalovirus immediate early promoter (CMV), were propagated in *Escherichia coli* (DH5α), purified by EndoFree Plasmid Maxi or Giga Kit (Qiagen, Hilden, Germany), and stored at −40 °C.

Labeling of block-copolymers and plasmid DNA (pTurboYFP) with Alexa Fluor647 N-succinimidyl ether and QD655 quantum dots ITK Streptavidin Conjugate (both — Invitrogen/Molecular Probes, Eugene, OR), respectively, for experiments with time-lapse microscopy was carried out as described earlier [4].

Polyplexes were prepared in sterile buffer solution (5% D-glucose, 5 mM HEPES, pH 7.4). Briefly, the polymer solution was added rapidly to the DNA and mixed by vortex followed by 20 min incubation at room temperature prior to use. Final concentration of plasmid DNA in polyplex solution was 20 μg per ml, N/P ratio was 20. Prepared polymeric nanoparticles were characterized in terms of its hydrodynamic diameters and ζ-potentials (Suppl. Table 1) by Dynamic Light Scattering Methods using ZetaPALS instrument (Brookhaven Instruments, Holtsville, NY) as described earlier [4].

2.2. Cell culture

Cloudman S91 mouse melanoma cells (clone M3) (ATCC-CCL-53.1) and the same cell line stably expressing the *H2A.Z-GFP* gene [29] encoding histone H2A.Z and GFP fusion protein under CMV-promoter, hereafter M3 and M3 H2A.Z-GFP, were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum. All cultured cells were grown at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. Transfection procedure

M3 cells were seeded onto 48-well plates, 12,000 cells per well, and incubated in DMEM/F12 medium supplemented with 10% fetal bovine serum for 24 h. After replacement of the cultural serum-containing medium, polyplex solutions were added to the cells to final DNA concentration of 0.5 μg ml^{-1}.

For live cell microscopy experiments, M3 H2A.Z-GFP cells were seeded onto POC minichambers (PeCon, Erbach, Germany), 150,000 cells per chamber, in 1.5 ml of DMEM/F12 medium with 10% fetal bovine serum, and cultivated at 37 °C and 5% CO_2. After 12 h, solution of PEI–PEG–MC1SP(NLS^+)-based polyplex nanoparticles, containing pTurboYFP plasmid DNA, was added to the cells to final DNA concentration of 0.5 μg ml^{-1}.

2.4. Time-lapse microscopy of live cells

Immediately after transfection POCmini chamber with cells was placed onto thermostatic stage (37 °C) of laser scanning microscope LSM-510 Meta NLO equipped with Plan-NEOFLUAR ×10/0.30 lens. Cell images were obtained using time-series lambda stack mode (32 images with time interval of 45 min between scanning) with excitation at 488 nm and 494–569 nm pass band for emission. On the next step, linear spectrum unmixing standard procedure was carried out. In case of transfection with double-labeled polyplexes at the end of time series imaging extra images of transfected cells were obtained using Plan-Apochromat ×63/1.4 Oil DIC lens and multitrack Z-stack mode. Alexa Fluor647-labeled block-copolymers were registered using excitation at 633 nm and 645–700 nm pass band for emission. QD655-labeled DNA, GFP in the cell nuclei and cytoplasmic YFP were visualized by an argon laser at excitation wavelengths of 458 nm, 488 nm and 514 nm, and 612–645 nm, 505–516 nm and 548–591 nm pass bands for emission, respectively.

2.5. Analysis of transfection efficiency

If M3 cells were transfected with pTurboYFP the cells were harvested after 24 h by treatment with 0.25% trypsin in Versene solution, precipitated by centrifugation at 200 ×*g* for 5 min, and resuspended in Versene solution. Analysis of YFP expression was performed using an Epics Altra Flow Cytometer (Beckman Coulter, Miami, FL). To discriminate between vital and apoptotic cells propidium iodide staining was carried out. Per sample, 1×10^4 gated events were collected.

In case of transfection with pCMV-NIS, the cells were washed after 24 h with HBSS, containing 10 mM HEPES, pH 7.4, and incubated for 1 h with the same solution containing 5.5 mM glucose and 1 μM $Na^{125}I$ (Isotope, Moscow, Russia) with activity of 0.1 μCi. In control wells the