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Lysosomal capturing of cytoplasmic injected nanoparticles by autophagy: An additional barrier to non viral gene delivery

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ABSTRACT

Autophagy or 'self-eating' is a process by which defective organelles and foreign material can be cleared from the cell's cytoplasm and delivered to the lysosomes in which degradation occurs. It remains an open question, however, whether nanoparticles that did *not* enter the cell through endocytosis can also be captured from the cytoplasm by autophagy. We demonstrate that nanoparticles that are introduced directly in the cytoplasm of the cells by microinjection, can trigger an autophagy response. Moreover, both polystyrene beads and plasmid DNA containing poly-ethylene-imine complexes colocalize with autophagosomes and lysosomes, as was confirmed by electron microscopy. This indicates that cytoplasmic capturing of nanoparticles can occur by an autophagy response. The capturing of nanoparticles from the cytoplasm most likely limits the time frame in which efficient nucleic acid delivery can be obtained. Hence, autophagy forms an additional barrier to non-viral gene delivery, a notion that was not often taken into account before. Furthermore, these findings urge us to reconsider the idea that a single endosomal escape event is sufficient to have the long-lasting presence of nanoparticles in the cytoplasm of the cells.

1. Introduction

In non-viral gene delivery, we aim to induce protein production by the cellular administration of plasmid DNA (pDNA) or mRNA that encodes the desired protein (Fig. 1). For gene therapy to be successful, the nucleic acids should reach the cytoplasm (in the case of mRNA) or the nucleus (in the case of pDNA) of the target cells. The ideal delivery system should guide the nucleic acids to their target cells, increase their cellular uptake and help the nucleic acids to escape from the endosomal compartment into the cytoplasm of the cells. Also, the possible degradation of the nucleic acids in the intracellular environment should be taken into account, as the intact sequence is necessary to maintain their biological activity [1].

For more than a decade, pharmacists, material scientists and biophysicists have been intensively studying the design, preparation and cell biological behavior of nanosized particles carrying nucleic acids [2–9]. The extensive research on nucleic acid delivery has revealed some general findings which should be taken into account when developing new gene delivery systems. It is for example accepted that positively charged nanoparticles are taken up better by cells [10]. As naked nucleic acids are negatively charged, they are complexed with cationic polymers or cationic liposomes to increase the cellular delivery. These complexes can take different endocytic pathways to enter the cells, dependent on their size and surface characteristics [11–14]. When nanoparticles fail to escape the endosomal compartment, eventual delivery to (and degradation in) the lysosomes will occur. Up to now, it is accepted that nanoparticles or naked nucleic acids that escaped from the endosomal compartment remain present inside the cytoplasm of the cells. We recently obtained evidence, however, that nanoparticles that are freely present inside the cytoplasm, can be captured inside cellular vesicles, possibly due to an autophagic response of the cells [15]. Autophagy or 'self-eating' is a process by which cells clear their cytoplasm from defective cell organelles and foreign material (Fig. 1) [16]. Initially, part of the cytoplasm is engulfed by a double-membraned structure (the phagophore, Fig. 1, step 7), which closes on itself to form an autophagosome (Fig. 1, step 8). These autophagosomes can receive additional input from endocytic vesicles before they eventually fuse with lysosomes to form an autolysosome, in which the sequestered cytoplasmic cargo is degraded (Fig. 1, step 9). Recently, it was shown for the first time that autophagy is a potential barrier in non-viral gene delivery after transfection of cells with nanoparticles. With electroporation, however, an autophagy response was not noted [17]. Also previously, we suggested that autophagy could be responsible for the long term endosomal entrapment of nanoparticles in Retinal Pigment Epithelial (RPE) cells [18]. In the current paper, we demonstrate the occurrence of autophagy on inert polystyrene beads and plasmid DNA containing poly-ethylene-imine (PEI) nanoparticles,

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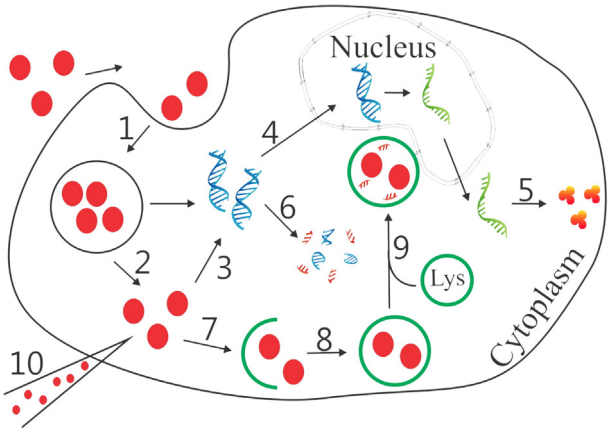

Fig. 1. Intracellular barriers to non-viral gene delivery. After cellular entry (1), nanoparticles have to escape from the endosomes (2) and release nucleic acids in the cytoplasm of the cells (3). Plasmid DNA then needs to travel to the nucleus (4), while mRNA can be translated to proteins in the cytoplasm of the cells (5). Clearly, degradation of nucleic acids should be avoided (6). When autophagy occurs, a phagophore isolates cytoplasmic material (7) and closes on itself to form an autophagosome (8). These fuse with lysosomes to form an autolysosome (9) in which the cytoplasmic cargo is degraded. In this research article, nanoparticles are directly delivered to the cytoplasm of the cells by microinjection (10).

that were directly introduced in the cytoplasm of the cells by microinjection (Fig. 1, step 10). Our findings suggest that, highly likely, endosomal escape alone is not sufficient to prevent cargo from eventual lysosomal degradation. The re-capturing of cytoplasmic nanoparticles in autophagosomes will limit the residence time of cytoplasmic cargo and could be a major determinant of why the majority of nanoparticles are entrapped in the endosomal compartment. This in turn limits the time frame during which mRNA can be translated in the cytoplasm, or pDNA can be delivered to the nucleus of the cells, thus limiting the efficiency of non-viral gene delivery systems.

2. Materials and methods

2.1. pDNA production & labeling

Heat competent *Escherichia coli* transformed with the gWIZ-GFP plasmid DNA (GeneTherapy Systems®, San Diego, California) were grown in LB medium with kanamycin at 37 °C for 20 h. pDNA was isolated and purified with the Qiafilter Plasmid Giga Kit (Qiagen®, Venlo, The Netherlands) according to the manufacturer's instructions. For colocalization experiments, pDNA was labeled with Cy5 (fluorescent red) using the *Label* IT® Cy™5 labeling kit (Mirus Bio Corporation, WI, USA) and purified by purification columns as supplied by the manufacturer. It should be noted that at the highest labeling density used, the Cy5-labeled pDNA can no longer induce GFP expression so it does not interfere with the colocalization measurements [19].

2.2. Polystyrene beads and pDNA/PEI polyplexes

Green fluorescent carboxylated polystyrene beads of 100 nm and red fluorescent carboxylated polystyrene beads of 40 nm were used (Molecular Probes®). These beads have a negative ζ-potential and were introduced into the cells by microinjection, uptake or electroporation as described below. As working solution, polystyrene beads were freshly diluted 1000 times in water and sonicated during 15 min before use. The diluted PS beads have an average concentration of 2×10^8 beads/μl. Upon injection, this results in between 8 and 20 injected beads per cell.

As pDNA nanoparticles we made use of pDNA complexed to 22 kDa linear poly-ethylene-imine (PEI) (kindly provided to us by Prof. Olivia Merkel, Wayne State University, USA). Complexes were prepared by adding a PEI polymer solution to an equal volume of a 0.4 μg/μl pDNA solution to obtain a nitrogen to phosphate ratio (N/P ratio) of 10, followed by vortexing the dispersion for 10 s. pDNA/PEI complexes were allowed to equilibrate at room temperature for 30 min prior to use. pDNA/PEI complexes have a positive zeta potential of 32 ± 5 mV and a size of 165 ± 15 nm in Hepes buffer. It should be noted that both for polystyrene beads and pDNA/PEI nanoparticles, the size and charge can change significantly in cell culture medium or optiMEM® [20].

For microinjection, 2 μl of beads (1000× diluted) or pDNA/PEI complexes were added to 6 μl of water or 1 μl 70 kDa TRITC–dextran and 5 μl of water. These solutions were filtered over a 450 nm filter (Millipore™, Overijse, Belgium) to avoid clogging of the microinjection needles. For electroporation, 10 μl of diluted beads or pDNA/PEI complexes were added to the cell in optiMEM®. For uptake experiments, 5 μl of diluted beads or pDNA/PEI complexes were incubated in cell culture medium for 2 h at 37 °C.

2.3. Cell culture conditions

HeLa cells (human epithelial cervical carcinoma cells, ATCC CCL-2, France) or HeLa cells stably expressing GFP-LC3 (kindly supplied to us by Prof. Felix Randow, Cambridge, UK) were cultured in phenol red free DMEM-F12 (Gibco-Invitrogen, Merelbeke, Belgium) containing 2 mM glutamine, 10% heat-inactivated fetal bovine serum (FBS) (Hyclone/Perbio, Thermo Fisher Scientific, Erembodegem-Aalst, Belgium) and 100 U/ml penicillin–streptomycin (Invitrogen-Gibco, Merelbeke, Belgium) at 37 °C in a humidified atmosphere containing 5% CO_2. HeLa cells were seeded in 35 mm glass bottom culture dishes (MatTek Corporation®) the day before use. For electron microscopy gridded coverslips were used, coated with Formvar and Gelatin as described before [21].

2.4. Microinjection

Microinjection with the polystyrene beads (green or red) or Cy5-pDNA/PEI nanoparticles (red) was performed with a Femtojet® microinjector and an Injectman® NI 2 micromanipulator (Eppendorf®) installed on a Nikon EZC1-si confocal laser scanning microscope. Injection needles were pulled from borosilicate glass capillaries (1.2 mm outer diameter and 0.94 mm inner diameter) with a P-1000 flaming/brown micropipette puller (Sutter Instruments, California, USA). Injections were performed in the cytoplasm, using polystyrene beads or pDNA/PEI complexes in HEPES buffer. If needed, injection solutions were supplemented with 2 mg/ml 70 kDa TRITC–dextran (Sigma-Aldrich®) to identify the place of injection. Typically, about 30–50 successful injections were performed during a 20 minute time period. After microinjection, we incubated the cells for an additional 24 h before taking fluorescence microscopy images. Labeling of the lysosomes was performed by transfecting the cells with CellLight™ Lysosomes-GFP BacMam 2.0, following the manufacturer's instructions (Invitrogen®). Colocalization with LC3 was evaluated using HeLa cells stably expressing GFP-LC3. The basal expression levels of non-injected cells expressing GFP-LAMP1 and GFP-LC3 can be found in the supplementary information (Suppl. Fig. 1). For live-cell imaging, the cells were placed on the microscope in a stage top incubation chamber (Tokai Hit, Shizuoka, Japan), set at 37 °C, 5% CO_2, and 100% humidity. Live-cell single particle tracking was performed on a custom-built laser widefield fluorescence microscope setup (TE2000-E inverted microscope) equipped with a Plan Apo VC 100X 1.4 NA oil immersion objective lens (Nikon Belux, Brussels, Belgium). Videos were acquired in NIS Elements (Nikon Benelux, Brussels, Belgium) at a frame speed of 30 frames per second and exposure times between 10 and 30 ms.

2.5. Electroporation and uptake

1.2×10^6 HeLa cells were electroporated at 260 V and 850 μF in 400 μl optiMEM containing the 100 nm green polystyrene beads.

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