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Gene delivery via the hybrid vector of recombinant adeno-associated virus and polyethylenimine

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ABSTRACT

The aim of this study was to investigate the cellular delivery mechanism of the hybrid vector comprising the recombinant adeno-associated virus (rAAV) and polyethylenimine (PEI). The rAAV vector, rAAV-rIns1-hInsM2-ΔEGFP, was fluorescently labeled with Cy3, a cyanine dye, and complexed with PEI. The interaction of the hybrid vector with the Huh7 hepatoma cells was monitored by confocal microscopy. Complexation of rAAV with PEI enhanced the transduction efficiency, which was decreased by pretreatment of the cells with sodium chlorate, an inhibitor of glycosaminoglycan sulfation, suggesting the roles of heparan sulfate proteoglycans (HSPG) in the uptake of the hybrid vector by the cells. Examination by flow cytometry and confocal microscopy demonstrated an enhanced interaction between the cells and the virus when complexed with PEI. Pretreatment with wortmannin or cytochalasin B significantly reduced the virus uptake by the cells, suggesting the involvement of phosphatidylinositol 3-kinase (PI3K) signaling and phagocytosis in the interaction between the cells and the hybrid vectors. Treatment of cells with the antioxidants, including L-ascorbic acid, δ-tocotrienol, or N-acetylcysteine (NAC), impaired the rAAV-PEI-mediated transduction. Results obtained in this study illustrated the involvement of PI3K/Akt signaling and the ROS production in gene delivery via the rAAV-PEI hybrid vector.

1. Introduction

Recombinant adeno-associated virus (rAAV), a non-enveloped and single stranded DNA parvovirus, is being developed as a promising gene delivery vector due to the lack of pathogenicity and low inflammatory responses (Mitchell et al., 2010). The prototype rAAV vectors based on AAV serotype 2 (AAV2) however exhibited a limited transduction efficiency and tissue tropism in mice (Mori et al., 2004). A number of non-viral vectors therefore have been incorporated with rAAV to enhance the transduction efficiency (Hsu and Yang, 2005b; Yang and Chao, 2003; Yang and Hsieh, 2001). Polyethylenimine, an organic macromolecule with high cationic charge densities, is capable of condensing the negatively charged nucleic acid, either DNA or RNA, to form stable nano-structured complexes. Consequently, these cationic macromolecules have been widely used as the non-viral vectors for gene delivery (Boussif et al., 1995a; Kichler et al., 2001). In our previous studies, complexation of rAAV with protamine or polycations, such as polyethylenimine, resulted in an enhancement of the transduction efficiency of rAAV (Hsu and Yang, 2005b; Yang and Hsieh, 2001). Employing this strategy, the glucose- and metabolically-regulated hepatic insulin gene therapy was obtained *in vivo* (Hsu et al., 2008). It was hypothesized that the polycationic macromolecules, conferring the positive charges and forming stable complexes with the rAAV particles, enabled the virus particles to bind to the negatively-charged cell surfaces, thus facilitated gene transfer.

Cellular proteoglycans such as heparan sulfate proteoglycans (HSPG) are known to play important roles in several cellular functions and serve as the viral receptor for AAV type 2, and therefore are involved in the uptake of virus particles, the fusion with the cell membrane, and the egress into the cytosol (Summerford and Samulski, 1998). Recent studies by Uhrig et al. also demonstrated that rAAV2 is internalized by the cells via the heparan sulfate proteoglycans (HSPG) and clathrin-dependent pathway (Uhrig et al., 2012). Our previous studies, on the other hand, indicated that the addition of PEI elevated the surface charge of Huh7 cells, suggesting the roles of electrostatic interaction of PEI in the enhancement of viral infection (Hsu and Yang, 2005a). To examine the roles of HSPG in transduction of the hybrid vectors, studies were therefore carried out here by treatment of the cells with sodium chlorate ($NaClO_3$), a potent inhibitor of glycosaminoglycan sulfation (Fadel and Eley, 2004; Halvorsen et al., 1998) which

Abbreviations: H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; HSPG, heparan sulfate proteoglycans; NAC, N-acetyl-L-cysteine; PI3K, phosphatidylinositol 3-kinase; PEI, polyethylenimine; rAAV, recombinant adeno-associated virus; ROS, reactive oxygen species.

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was employed to examine the roles of HSPG in the rAAV-PEI mediated gene delivery. To visualize the internalization of rAAV-PEI complexes, the rAAV virions were fluorescently labeled, and examined for the entry into the cytosol or nucleus compartment of the cells by fluorescence microscopy, followed by semi-quantification by the Image-Pro Plus software (Media Cybernetics, Inc.). To examine the dependence of the entry mechanism and the uptake of rAAV-PEI on the (PI3K)/Akt signaling, previously shown to be important in the virus entry (Feng et al., 2011), cells were treated with wortmannin, a potent inhibitor of phosphatidylinositol 3-kinase (PI3K) phosphorylation, and cytochalasin B, a phagocytosis inhibitor, prior to transduction with rAAV-PEI, followed by microscopic examination of the virus entry into the cytosol or the nucleus.

The reactive oxygen species (ROS), known to be intracellular signaling molecules and act as the defense mechanisms against microorganisms such as bacteria or viruses (Puertollano et al., 2011), are involved in maintaining the cellular redox homeostasis (Rosanna and Salvatore, 2012). It is unclear if the ROS generation is involved in the hybrid vector-mediated gene delivery, and the roles of the oxidative stress in the enhancement of PEI on rAAV-mediated gene transfer remain to be determined. We hypothesized that the enhancement of the transduction efficiency of the hybrid vector may be attributed to the production of oxidative stress, induced by the charges of polycationic PEI. Continuing the previous studies on rAAV-mediated gene therapy (Hsu and Yang, 2005b), we attempt to examine in this study the roles of the reactive oxygen species (ROS) in gene transfer via the hybrid rAAV-PEI nanocomplexes. Vitamin C and vitamin E are commonly used dietary antioxidants that improve different immune functions and exhibit the protection from infections caused by the bacteria or viruses (Puertollano et al., 2011). Vitamin C is an effective antioxidant that reacts with a wide spectrum of radicals to give ascorbate and dehydroascorbate, and has been used to inhibit ROS production in chemotherapy (Fukumura et al., 2012). Vitamin E, made up of four tocopherols (alpha, beta, gamma, delta) and four tocotrienols (alpha, beta, gamma, delta), on the other hand, is known to be a potent antioxidant and radical scavenger in chemical and biological systems (Brigelius-Flohe and Traber, 1999; Cerecetto and Lopez, 2007; Winterbourn, 2008). Tocotrienols have been reported to possess excellent antioxidant activity *in vitro* and suppress ROS production more efficiently than tocopherols (Schaffer et al., 2005). N-acetylcysteine (NAC), a sulfidryl-containing thiol compound, is a potent antioxidant and directly as a free radical scavenger (Berniakovich et al., 2012). These chemicals were employed to investigate the effect of antioxidants and to determine the roles of ROS in rAAV-PEI mediated gene delivery.

Studies were hereby carried out employing the previously constructed rAAV vector, rAAV.rIns1.InsM2.ΔEGFP that harbors the furin-mutated human insulin gene, driven by rat insulin I gene promoter (German, 1993; German et al., 1990; German and Wang, 1994), and the open reading frame of the enhanced green fluorescence protein (EGFP), under the control of the immediate early (IE) gene promoter of human cytomegalovirus (CMV). The recombinant viruses were prepared, isolated, purified, and complexed with PEI as previously described (Hsu et al., 2008; Hsu and Yang, 2005b). Huh7 hepatoma cells were incubated with rAAV or rAAV-PEI complexes, either in the absence or presence of the antioxidants, followed by flow cytometric analysis of the expression of the EGFP gene.

Our experimental results showed that complexation of rAAV with PEI induced the generation of ROS and enhanced the rAAV-mediated transduction efficiency. Treatment of cells with the antioxidants, such as vitamin C, vitamin E, or N-acetylcysteine, decreased the transduction efficiency of the hybrid vector. These data demonstrated the roles of ROS in gene delivery mediated by the rAAV-PEI hybrid vector.

2. Materials and methods

2.1. Materials

Polyethylenimine (branched, 25 K), L-ascorbic acid (vit. C), δ-tocotrienol (vit. E), N-acetylcysteine (NAC), and most chemicals were obtained from Sigma–Aldrich (St. Louis, MO). All cell culture materials were purchased from HyClone (Logan, UT). 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) and dihydroethidium (DHE) were both obtained from Molecular Probe® Invitrogen.

2.2. Methods

2.2.1. Preparation of rAAVs and fluorescence labeling by Cy3 fluorochrome

Recombinant AAV (rAAV.rIns1.InsM2.ΔEGFP) was prepared by the non-viral method as previously described (Hsu et al., 2008; Hsu and Yang, 2005b). Briefly, rAAV was grown in 293T cells, released from the cells by four freeze–thaw cycles, were subjected to ultracentrifugation in the CsCl gradients and subsequent dialysis. To visualize the binding of the virus onto the cells, rAAV was labeled with Cy3 fluorochrome employing the Cy3™ Bis-Reactive Dye (PA23000; Amersham Pharmacia Biotech), following the manufacturer's protocol. After a 30-min labeling reaction, Cy3-labeled virus was purified by gel filtration through a Sephadex G-25 column and kept at 4 °C before use. The genomic titers of the virus were determined by DNA dot-blot hybridization with [α-^{32}P]-dCTP-labeled DNA probes, as previously described (Hsu et al., 2008; Hsu and Yang, 2005b). Complexation of rAAV with PEI was performed by mixing the virus particles with PEI-25 K (2 μg/ml) prior to addition to the cells.

2.2.2. Effect of PEI on the adsorption and internalization of rAAV by the Huh7 cells

To examine the effect of PEI on the adsorption of rAAV onto the cells *in vitro*, 5×10^5 Huh7 cells per well in the 12-well plates were treated with Cy3-labeled rAAV, complexed with or without PEI (2 μg/ml), and incubated at 37 °C for 2 h. Cells were washed three times with phosphate-buffered saline (PBS) and analyzed with a FACSCalibur flow cytometer (BD Biosciences).

2.2.3. Examination of cell-virus interaction by fluorescence microscopy

To assess the amount of the adsorption or internalization of Cy3-rAAV, Huh7 cells were grown at 1×10^5 per well on the poly-L-lysine coated cover slips in the 6-well plates. Cells were pre-treated with 3 μM wortmannin or 10 μM cytochalasin B for 1 h and washed with PBS. Fresh culture medium was added, and cells were infected with Cy3-rAAV or Cy3-rAAV-PEI (MOI 2000), and incubated at 4 °C for 30 min, followed by washes with cold PBS to remove the unbound virions, and incubation at 37 °C for 2 h. Cells were then fixed with ice-cold methanol, mounted with Clearmount (Zymed, San Francisco), and observed under a Zeiss Axiophot 2 fluorescence microscope. Quantitative fluorescence imaging analysis was performed using the ImagePro Plus 4.5 software (Media Cybernetics, Inc., MD, USA).

2.2.4. Effect of $NaClO_3$ on the binding of rAAV

To investigate the potential functions of cell-surface proteoglycans during virus adsorption, $NaClO_3$ was employed to inhibit the sulphation of proteoglycans. Huh7 cells at 1×10^5 cells/well were cultured in RPMI medium, containing 2% fetal bovine serum (FBS), in the 12-well plates and treated with 35 mM $NaClO_3$ for 48 h,