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Enhanced cellular secretion of AAV2 by expression of foreign viral envelope proteins

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ABSTRACT

Recombinant adeno-associated virus (rAAV), a non-enveloped virus, is widely used in gene therapy clinical trials because it does not cause human disease, transduces both dividing and non-dividing cells, and mediates stable transgene expression for years in post-mitotic tissue. Extension of clinical use of rAAV is, however, considerably hampered by difficulties involved in large-scale production of the virus particles. For several serotypes of rAAV these difficulties often arise from the fact that assembled virus particles mainly stay inside of packaging cells, inevitably requiring lysis of cells to harvest virus particles and consequentially complicating downstream purification processes. Here, we show that introduction of foreign viral envelope protein genes, encoding for either VSVG or rabiesG, into packaging cells can remarkably enhance cellular secretion of rAAV2, the AAV serotype most often used in clinical applications. In the presence of the foreign genes, up to 49% of transducing rAAV2 particles were secreted. However, such great enhancement was not observed for rAAV3. Our experimental tests with exosome inhibitors indicated that VSVG-mediated cellular secretion of rAAV2, unlike rabiesG-mediated one, heavily relies upon cellular pathways involving exosomes. Ultimately, an improved understanding of rAAV secretion mechanisms may simplify the production and purification processes for large-scale batches of the virus.

1. Introduction

Recombinant adeno-associated virus (rAAV) has been increasingly used as a gene delivery vehicle for preclinical genetic studies and gene therapy clinical trials [1–6]. Excitingly, the first rAAV-based gene therapy drug, Glybera, which targets lipoprotein lipase deficiency (LPLD), has recently been approved by the European Commission [7]. The increased use and clinical success of rAAV is due to several characteristics that make these vectors favorable for gene therapy [5,6,8]. rAAV vectors can effectively transduce both dividing and non-dividing cells and have not been associated with any human disease. In addition, there are various AAV serotypes, each of which has a distinct tropism to specific cell types and tissues. However, extension of therapeutic use of rAAV vectors currently suffers, in part, from difficulties with large-scale viral production [9–13]. Assembly of rAAV particles occurs inside of cells for several serotypes and thus, conventional rAAV production methods often involve an initial cell lysis step to release intracellular viral particles as well as cellular impurities, such as proteins, nucleic acids and lipids that must be eliminated. The lysis step complicates downstream purification processes and also increases the overall cost of viral production since lysing cells prevents that cells keep producing more viral particles. Alternatively, the development of a process in which growing cells continuously and efficiently secrete rAAV particles into the media, would simplify the viral production by eliminating the cellular lysis step.

A well-studied viral envelope protein, envelope protein G from vesicular stomatitis virus (VSVG), is known to be trafficked to the plasma membrane via transport vesicles after being intracellularly synthesized [14,15]. Based on the finding that VSVG can stimulate the formation of intracellular transport vesicles [15], we hypothesized that the expression of VSVG may trigger active secretion of rAAV particles. In this study, we show that introduction of genes encoding the envelope proteins of VSV and a closely

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related rabies virus (VSVG and rabiesG) into packaging cells significantly enhances the secretion of rAAV2 particles, the serotype most often used in clinical trials. In the presence of genes encoding VSVG or rabiesG, up to 49% of assembled transducing rAAV2 particles were secreted from packaging cells. Our experimental tests with exosome inhibitors further indicated that the VSVG-mediated cellular secretion of rAAV2 particles relies upon cellular pathways involving exosomes—vesicles of 40 to 100 nm in diameter that are produced from the plasma membrane of animal cells [16]. In contrast, rabiesG-mediated secretion of the virus particles was independent of exosome-involving cellular pathways. However, introduction of VSVG did not significantly increase secretion of rAAV3 particles indicating that the type and amount of envelope protein used for secretion enhancement must be optimized for individual AAV serotypes.

As a proof-of-principle, we demonstrated that we could enhance secretion of rAAV2, a non-enveloped virus, by expressing proteins from enveloped viruses. If a general method to increase secretion of rAAV in a serotype-independent fashion is further developed in the near future from better understanding of the relevant cellular mechanisms in combination with construction of virus packaging cells in suspension, this could lead to a continuous process to produce rAAV particles at a large scale and a lower cost.

2. Materials and methods

2.1. Cell lines

Cell lines were cultured at 37 °C and 5% CO_2, and unless otherwise mentioned, were obtained from the American Type Culture Collection (Manassas, VA). HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). CHO K1 and CHO pgsA cells were cultured in F-12K medium (ATCC) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen). CHO Lec1 cells were cultured in MEM-alpha medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen).

2.2. Viral production and titering

Recombinant AAV2 vectors expressing GFP under the control of a CMV promoter were packaged in HEK293T cells using the calcium phosphate transfection method [17,18]. Briefly, HEK293T cells grown in a 10 cm plate were transfected with 9 μg pXX2 plasmid (containing AAV2 *rep*/*cap* genes), 9 μg double-stranded pAAV-CMV-GFP plasmid (containing CMV-GFP between AAV2 ITRs), and 7 μg pHelper plasmid for the control case and the other cases. To increase the secretion of packaged rAAV particles compared with the control case, transfections were additionally supplemented only with various amounts of plasmid containing a VSVG or rabiesG gene (from a major variant of mouse-brain-adapted rabies strain: CVS-N2c virus) [19,20]. Supernatant and cell lysate samples containing assembled rAAV particles were collected 48 h post-transfection. Cell lysate samples were prepared by three freeze-thaw cycles, followed by centrifugation to remove cellular debris. The plasmid containing the rabiesG gene was a kind gift from Dr. Matthias Schnell (Thomas Jefferson University). For production of VSVG-pseudotyped HIV-1 based lentiviral vectors 10 μg pFUGW, 5 μg pMDLg/pRRE, 1.5 μg pRSV-Rev and 3.5 μg pcDNA3 IVS VSV-G plasmid were introduced into HEK293T cells in a 10 cm plate via the calcium phosphate transfection method. Lentivirus supernatant was harvested at two and three days post-transfection and concentrated by ultracentrifugation. Genomic titers of DNase-resistant rAAV physical particles were determined via quantitative PCR as previously described [18]. Transducing titers of rAAV particles were determined by transducing 2.6×10^5 HEK293T cells and monitoring the GFP expression of the cells. The percentage of GFP-positive cells was assessed 48 h post-transduction using a Beckman-Coulter Cytomics FC 500 flow cytometer (Brea, CA).

2.3. Inhibition of exosomes and cell imaging

For inhibition of exosomes, cells were treated with either 5-(*N*,*N*-dimethyl)amiloride (Enzo Life Sciences, Farmingdale, NY) or methyl-beta-cyclodextrin (Sigma Aldrich, St. Louis, MO) at the concentration of 25 μg/ml from 24 h post-transfection until virus harvesting 48 h post-transfection. To monitor cell morphology during AAV packaging and expression of viral envelope proteins, cells were imaged at different times post-transfection using a Zeiss Axio Observer A1 inverted microscope.

2.4. In vitro transduction analysis

To determine the relative transduction efficiency and heparin binding properties of rAAV vectors, CHO K1, CHO pgsA, and CHO Lec1 cells were plated at a density of 2.5×10^4 cells per well 24 h prior to transduction. Cells were transduced with GFP-encoding vectors at a range of genomic multiplicity of infections (gMOIs) 100–1000 [21]. The percentage of GFP-positive cells was assessed 48 h post-transduction using a Beckman-Coulter Cytomics FC 500 flow cytometer.

2.5. Neutralization of rAAV vectors with intravenous immunoglobulin (IVIG)

HEK293T cells were plated at a density of 1.5×10^4 cells per well 24 h prior to transduction. rAAV vectors that had been harvested from the cell culture supernatant were incubated for 1 h with varying concentrations of heat-inactivated Gamimune N, 10% Human IVIG (Bayer, Elkhart IN) prior to transduction, and the cells were then transduced with the GFP-encoding vectors at gMOI of 2000. The percentage of GFP-positive cells was assessed 48 h post-transduction using an ImageXpress Micro Cellular Imaging and Analysis System (Molecular Devices, Sunnyvale, CA) and MetaXpress Image Analysis Software (version 3.1.0) with the Multi Wavelength Cell Scoring Application Module (Molecular Devices).

3. Results

3.1. Expression of foreign envelope proteins significantly increased secretion of rAAV2 particles from packaging cells

To determine whether cellular secretion of AAV2 can be increased by expression of envelope proteins from other viruses, we introduced the genes encoding VSVG or rabiesG into HEK293T cells during transfection of AAV packaging plasmids. As more plasmid containing a VSVG or rabiesG gene was added into packaging cells, the corresponding viral envelope proteins were expressed at a higher level (Figs. S1 S3 in Supplementary information). Without the genes encoding the foreign envelope proteins, only $4.62 \times 10^8 \pm 2.33 \times 10^7$ DNase-resistant genomic rAAV2 particles, approximately 0.2% of the total virus yield (Fig. 1A), and $5.47 \times 10^5 \pm 3.40 \times 10^4$ transducing rAAV2 particles, approximately 15% of the transducing virus yield, were released from a 10 cm dish of packaging cells into the cell culture supernatant (Fig. 1B). Therefore, most assembled viruses stayed inside of the cells in the absence of the foreign proteins. In comparison, introduction of the VSVG gene into packaging cells greatly increased the