Short communication

Transduction of striatum and cortex tissues by adeno-associated viral vectors produced by herpes simplex virus- and baculovirus-based methods

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Article history:
Received 11 August 2011
Received in revised form 28 September 2011
Accepted 4 October 2011
Available online 12 October 2011

Keywords:
Adeno-associated virus
Herpes simplex virus type I
Baculovirus
Gene delivery
Intracranial injection

ABSTRACT

Recombinant adeno-associated virus (AAV) vectors can be engineered to carry genetic material encoding therapeutic gene products that have demonstrated significant clinical promise. These viral vectors are typically produced in mammalian cells by the transient transfection of two or three plasmids encoding the AAV rep and cap genes, the adenovirus helper gene, and a gene of interest. Although this method can produce high-quality AAV vectors when used with multiple purification protocols, one critical limitation is the difficulty in scaling-up manufacturing, which poses a significant hurdle to the broad clinical utilization of AAV vectors. To address this challenge, recombinant herpes simplex virus type I (rHSV-1) and recombinant baculovirus (rBac)-based methods have been established recently. These methods are more amenable to large-scale production of AAV vectors than methods using the transient transfection of mammalian cells. To investigate potential applications of AAV vectors produced by rHSV-1- or rBac-based platforms, the in vivo transduction of rHSV-1- or rBac-produced AAV serotype 2 (AAV2) vectors within the rat brain were examined by comparing them with vectors generated by the conventional transfection method. Injection of rHSV-1- or rBac-produced AAV vectors into rat striatum and cortex tissues revealed no differences in cellular tropism (i.e., predominantly neuronal targeting) or anteroposterior spread compared with AAV2 vectors produced by transient transfection. This report represents a step towards validating AAV vectors produced by the rHSV-1- and the rBac-based systems as promising tools, especially for delivering therapeutic molecules to the central nervous system.

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Adeno-associated virus (AAV), a parovirus with a 4.7 kb single-stranded DNA genome containing two genes (rep and cap), has been explored in clinical trials for the treatment of a broad range of diseases, including Parkinson’s disease (Fiandaca and Bankiewicz, 2010), hemophilia B (by delivering the Factor IX gene) (Kay et al., 2000), and Leber’s congenital amaurosis (Bainbridge et al., 2008; Maguire et al., 2008). AAV typically has the capability to deliver genes to both dividing and non-dividing cells, such as muscle (Fisher et al., 1997), brain (Kaplitt et al., 1994), and retina (Flannery et al., 1997) cells. AAV does not elicit toxic or immune responses in animal models and does not suffer from rapid transcriptional silencing, further enhancing its potential as a candidate for the treatments of a variety of human diseases.

One critical challenge for bringing AAV vectors into clinical trials involves manufacturing sufficient quantities of highly purified vectors at acceptable costs. A conventional method used widely for producing all AAV serotypes is the transient transfection of three plasmids, including an AAV helper plasmid containing rep and cap genes in trans, the adenoviral helper gene plasmid required for vector replication, and a plasmid carrying a gene of interest flanked by AAV inverted terminal repeats (ITRs) (Wright, 2009). The transient transfection method, which has been shown to produce vectors capable of efficient transduction of mammalian cells, is typically performed by coprecipitation of the three plasmids using calcium phosphate (Graham and van der Eb, 1973), polycationic polymers (e.g., polyethyleneimine) (Boussif et al., 1995), or cationic lipids (Felgner et al., 1987). This method can eliminate contamination of the AAV stocks with a helper virus (e.g., adenovirus), which is typically required for AAV replication, during the process of production. It can also be adapted to produce AAV vectors of different serotypes. However, a major limitation of this method is the difficulty of scaling up transient transfections to produce sufficient vectors for large-scale clinical applications (Merten et al., 2005).

To address this limitation of the transient transfection method, large-scale viral vector production technologies based on use of a recombinant herpes simplex virus (rHSV) type 1 (Conway et al., 1999; Wu et al., 2002) or a recombinant baculovirus (rBac) (Blissard, 1996; Urabe et al., 2002) have been established. The rHSV-1 platform is based on the use of a replication-defective rHSV-1
containing the AAV rep and cap genes and a cell line carrying the recombinant AAV (rAAV) proviral genome, which is generated by the stable transformation of a plasmid carrying the gene of interest. The rHSV-1 serves as a helper virus for rAAV replication, resulting in viral yields (i.e., total viral quantities) that are approximately two orders of magnitude higher than those acquired by the conventional transfection method (Wu et al., 2002). The rBac platform, which is based on use of the Autographa californica nuclear polyhedrosis virus, has also been adapted to produce rAAV in invertebrate cells on a large scale (Urabe et al., 2002). This strategy involves engineering the AAV rep/cap genes and a transgene into three recombinant baculoviruses to generate a rep-rBac, a cap-rBac, and a transgene-rBac. Coinfection of these recombinant baculoviruses into insect cells (e.g., Spodoptera frugiperda cells) resulted in a 10-fold higher yield of AAV particles than that obtained with the transient transfection method using 293 cell lines (Urabe et al., 2002). Both rHSV-1- and rBac-based platforms can be alternatives to the transient transfection method, and they produce much larger quantities of AAV vectors, making them potentially more suitable for human gene therapy. However, for AAV vectors produced by the rHSV or the rBac systems to be adopted widely, it is crucial to determine whether the vectors produced by these methods have predictable infectivity such as tropism and vector spread, comparable to those of transient transfection.

The in vivo performances of the AAV vectors produced by these different methods, which have not been evaluated previously in a single study, were assessed in the rat brain. Each AAV vector, containing AAV serotype 2 (AAV2) capsids and encoding green fluorescence protein (GFP), was injected into the rat striatum and cortex. These tissues are potential target regions for the delivery of therapeutic molecules for the treatment of neurological disorders such as Parkinson's disease, Huntington's disease, and stroke. After delivery of the AAV vectors generated by each production method, cellular tropism and the spread of the transgene expression were examined. This study demonstrates the features of AAV2 vectors manufactured by rHSV-1- and rBac-based production platforms, which make them potentially suitable for applications in human gene therapy.

AAV serotype 2 vectors were produced by three different methods: (i) the transient transfection method using AAV 293 cell lines (AAV2-293), (ii) a recombinant herpes simplex virus type I (rHSV-1)-based method (AAV2-rHSV-1), and (iii) a recombinant baculovirus (rBac)-based method (AAV2-rBac). For the transient transfection method, recombinant AAV2 (rAAV2) vectors carrying cDNA encoding GFP were packaged and purified by CsCl-gradient ultracentrifugation followed by PD-10 (GE Healthcare Life Sciences, Pittsburgh, PA) desalting filtration, as described previously (Koerber et al., 2006; Maheshri et al., 2006). For rHSV-1- and rBac-induced packaging, the rAAV vectors carrying cDNA encoding GFP under a CMV promoter were purchased from Vector Gene Technology Company (Beijing, China) and Virovec (Hayward, CA, USA). These companies have produced AAV vectors for previous studies on rHSV-1-induced systems (Tian et al., 2011; Wu et al., 2002) and rBac-induced systems (Chen, 2008; Urabe et al., 2002). Viral vectors produced by the use of the rBac-induced system (i.e., those from Virovec) were purified by the same method as those produced using the transient transfection method. Viral vectors generated using the rHSV-1-induced system (i.e., those from the Vector Gene Technology Company) were purified using ion exchange column chromatography followed by filtration through a molecular sieve (Sephacryl S-200 High Resolution column; Amersham Pharmacia Biotech, Piscataway, NJ). DNase-resistant genomic titers were determined by quantitative PCR (QPCR; Mini Opticon, Bio-Rad, Hercules, CA). As a result, the transient transfection method produced the highest genomic titers (3.99 × 10^{12} ± 1.45 × 10^{12} vg (viral genomes)/mL), and the rHSV-1- and rBac-based methods produced 1.29 × 10^{12} ± 2.23 × 10^{11} vg/mL and 1.35 × 10^{11} ± 4.03 × 10^{11} vg/mL, respectively. Additionally, transduction of HEK293T cells lines by the transient transfection-, rHSV 1-, and rBac-based methods, generated 1.31 × 10^{10} ± 1.33 × 10^{10}, 4.22 × 10^{9} ± 2.53 × 10^{9}, and 6.95 × 10^{9} ± 8.67 × 10^{9} transducing units (TU)/mL, respectively. There were no statistical differences in either the genomic titers or transducing units among the viral vectors produced by the three different methods (p > 0.05).

The heparin affinities of the AAV vectors produced by the different packaging methods were assessed to determine whether any of the AAV vectors could possibly use heparan sulfate as its primary receptor (Fig. 1A). Approximately 10^{11} purified genomic particles of virus were loaded onto a 1 mL Hitrap heparin column (GE Healthcare Life Sciences), which had been equilibrated with Tris buffer (50 mM, pH 7.5) containing 150 mM NaCl. Elutions were performed with a series of increasing NaCl concentrations (150–750 mM and 1 M), and infectious viral titers of the HEK293T cells were determined by flow cytometry for each fraction. Similar to previous reports (Maheshri et al., 2006), all AAV vectors eluted in a sharp peak with 450–550 mM NaCl, indicating that none of the production methods altered the ability of the AAV to bind heparin.

The capacity of each AAV vector to transduce cultured cells, including HEK293T, HeLa, B16F10, and CHO-K1 cells, was assessed next (Fig. 1B). One day prior to infection, the cells

Fig. 1. Characterization of each AAV vector produced by transfection of 293 cells and rBac- and rHSV-1-based methods. (A) Heparin column chromatograms are shown for the comparison of the heparin affinities of the viral vectors. “Fraction virus eluted” (vertical axis) indicates the ratio of the infectious viral titer present in a designated elution to the infectious viral titer present in the solution prior to column loading. (B) In vitro transduction abilities of the AAV2 vectors are indicated. AAV-GFP vectors produced by transient transfection of 293 cells, the rHSV-1-based method, and the rBac-based method were used to infect HEK293T cells (genomic MOI: 10,000), HeLa cells (MOI 10,000), B16F10 cells (MOI 10,000), and CHO-K1 cells (MOI 10,000). The percentages of GFP-positive cells were quantified by flow cytometry.
were seeded onto 24-well tissue culture plates at a density of 20,000–30,000 cells/well, and the cells were infected on day 1 by each AAV vector at $10^4$ genomic multiplicity of infection (MOI). At 48 h post-infection, the resulting transduction efficiencies (i.e., the fraction of cells that expressed GFP) of each rAAV vector was quantified with a Becton Dickinson FACS Caliber (Yonsei University College of Medicine Medical Research Center). For HEK293T infection, AAV2 vectors produced from the rHSV-1 method exhibited the highest transduction efficiency (85.6%) compared with the other AAV2 vectors. The AAV2 vectors generated by the rBac-system showed the best performances in cellular transduction in several other cell lines (HeLa, B16F10, and CHO-K1). However, all of the AAV vectors yielded over 50% transduction efficiencies in all cell types, implying that the biological properties of the AAV2 vectors produced by the three helper systems are indistinguishable, which is consistent with a previous study (Urabe et al., 2002).

To investigate the in vivo performances of the AAV vectors produced by the three different methods, the same number of genomic particles of each AAV2 vector (3 μL of $1 \times 10^9$ vg/μL for a total of $3 \times 10^9$ viral particles) were injected stereotaxically into the striatum (anteroposterior [AP], +0.2 mm; mediolateral [ML], ±3.5 mm; and dorsoventral [DV], −4.5 mm from the skull) and cortex ([AP], +2.7 mm; [ML], ±2.5 mm; and [DV], −0.5 mm from the skull) of adult female Fischer 344 rats (150 g, 6 weeks old). Three weeks post-injection, robust GFP expression was observed in all animals, with a wide distribution around the injection sites in the striatum and the cortex (Figs. 2 and 3). GFP expression was detected by a primary rabbit anti-GFP antibody (diluted 1:2000; Invitrogen, Carlsbad, CA). Primary mouse anti-neuron-specific nuclear protein (NeuN, diluted 1:200; Chemicon International, Temecula, CA) and guinea pig anti-glial fibrillary acidic protein (GFAP, diluted 1:1000; Advanced ImmunoChemical, Long Beach, CA) antibodies

Fig. 2. In vivo gene delivery and cellular tropism of GFP-expressing AAV2 vectors within the rat striatum. (A) Representative images of infections with AAV2 vectors generated by transient transfection-, rBac-, and rHSV-1-based methods within the rat striatum show similar levels of GFP expression (green) at 3 weeks post-injection (scale bar: 180 μm). (B) Representative images of cell types residing adjacent to GFP-expressing cells within the rat striatum demonstrate the equivalent tropism for each AAV2 vector: (B) NeuN+ neurons (blue)/GFP+ cells (green); (C) GFAP+ astrocytes (red)/GFP+ cells (green) (scale bars: 50 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
were utilized to identify cell types such as neurons and glial cells (represented by astrocytes). The corresponding secondary antibodies, which were labeled with Alexa Fluor 488, 546, and 633 and diluted 1:250, were used for detection. The sections containing regions exhibiting GFP expression were collected, and the anteroposterior spread of each AAV vector type that had been injected into the striatum and the cortex was quantified to evaluate the in vivo performance of each AAV vector. As with in vitro transduction, AAV vectors produced by different methods exhibited similar performances following cranial delivery when assayed for cell targeting and spread within the brain.

In both the striatum and the cortex, the cellular tropism of AAV2-rHSV-1 and AAV2-rBaculovirus vectors remained identical to that of vectors produced by the transient transfection method using a 293 cell line (Figs. 2 and 3). Transduction (monitored by GFP expression) primarily occurred in cells that stained positive for the neuronal marker NeuN, with minimal infection of glial cells expressing the astrocytic marker GFAP. These observations indicate that rAAV2 vectors created by the rHSV-1- and rBac-systems are highly efficient in vivo, and their performances are not affected substantially by these alternative AAV production methods. This is consistent with the result that rAAV2 replicated by the HSV-1 and baculovirus systems efficiently transduced photoreceptor cells in mice, which are typically permissive to rAAV2 produced by the transient transfection method. This indicates similar physical and biological properties of the AAV vectors produced by the three procedures (Urabe et al., 2002; Zhang et al., 1999).

Additionally, there were no statistical differences in viral infection spread along the parts of anteroposterior axis accessed by viral vector infection among the three different viral preparations (Fig. 4). The gene delivery properties (anteroposterior spread and cellular tropism) of the AAV2 vectors produced by transient
transfection and the rHSV-1 and rBac systems are comparable in in vivo models, suggesting that the in vivo performances of these viral vectors are not substantially affected by different viral production systems. Interestingly, free diffusion of each vector was somewhat hindered in the cortex (∼500–1000 μm) relative to the striatum (∼1000–2000 μm).

In conclusion, this study describes the in vivo performance of rAAV vectors produced by three different methods: (i) the transient transfection of mammalian cells with three plasmids, (ii) the rHSV-1 helper system, and (iii) the rBaculovirus helper platform. The rHSV-1- and rBaculovirus-based replication platforms did not influence either the in vitro or in vivo performances of each vector. Robust production methods for rAAV vectors will certainly enhance the applicability of AAV as a gene delivery vehicle, specifically for human gene therapy applications, which typically require large amounts of viral vectors. Neuronal tropism of the AAV vectors produced by the rHSV-1 or the rBaculovirus system demonstrates that these two platforms can be alternatives to the conventional transfection approach, especially for investigating treatments for neurological disorders.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

This work was supported by Advanced Biomass R&D Center (ABC) of Korea (Grant No. 2010–0029734) and by the Human Resources Development of the Korea Institute of Energy Technology Evaluation and Planning (KETEP) grant funded by the Korea government Ministry of Knowledge Economy (No. 20104010100500).

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