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Application of polyploid adeno-associated virus vectors for transduction enhancement and neutralizing antibody evasion

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ABSTRACT

Adeno-associated virus (AAV) vectors have been used successfully in clinical trials for patients with hemophilia or blindness, but pre-existing neutralizing antibodies (Nab) are common in the general population and exclude many patients from clinical trials. Exploration of effective strategies to enhance AAV transduction and escape from Nab activity is still imperative. Previous studies have shown the compatibility of capsids from AAV serotypes and homology of recognition sites of AAV Nab located on different capsid subunits from one virion. In this study, we co-transfected AAV2 and AAV8 helper plasmids at different ratios (3:1, 1:1 and 1:3) to assemble haploid capsids and study both their transduction efficiency and Nab escape activity. After muscular injection, all of the haploid viruses induced higher transduction than their parental AAV vectors (2- to 9-fold over AAV2), with the highest of these being the haploid vector AAV2/8 3:1. After systemic administration, a 4-fold higher transduction in the liver was observed with haploid AAV2/8 1:3 than that with AAV8 alone. We then packaged the therapeutic factor IX cassette into haploid AAV2/8 1:3 capsids and injected them into FIX knockout mice *via* the tail vein. Higher FIX expression and improved phenotypic correction were achieved with the haploid AAV2/8 1:3 virus vector when compared to that of AAV8. Additionally, the haploid virus AAV2/8 1:3 was able to escape AAV2 neutralization and did not increase capsid antigen presentation capacity when compared to AAV8. To improve the Nab evasion ability of the haploid virus, we produced the triploid vector AAV2/8/9 by co-transfecting AAV2, AAV8 and AAV9 helper plasmids at a ratio of 1:1:1. After systemic administration, a 2-fold higher transduction in the liver was observed with the triploid vector AAV2/8/9 than that with AAV8. Nab analysis demonstrated that the triploid AAV2/8/9 vector was able to escape Nab activity from mouse sera immunized with parental serotypes. These results indicate that polyploid viruses might potentially acquire advantages from parental serotypes for enhancement of AAV transduction and evasion of Nab recognition without increasing capsid antigen presentation in target cells. Polyploid AAV vectors can be generated from any AAV serotype, whether natural, rational, library derived or a combination thereof, providing a novel strategy that should be explored in future clinical trials in patients with neutralizing antibodies.

1. Introduction

Adeno-associated virus (AAV), a non-pathogenic parvovirus that requires a helper virus for efficient replication, is utilized as a viral vector for gene therapy because of its safety and simplicity. AAV has a broad host and cell-type tropism capable of transducing both dividing and non-dividing cells. To date, 12 AAV serotypes and > 100 variants have been identified [1,2]. Different serotype capsids have different infectivity rates in tissue and cultured cells, which depend on the primary receptor and co-receptors on the cell surface, or on the intracellular trafficking pathway itself. The primary receptors of some serotypes of AAV have been found, such as heparin sulfate proteoglycan (HSPG) for AAV2 and AAV3, and N-linked sialic acid for AAV5 [3,4]. The primary receptors of AAV7 and AAV8 have not been identified. Interestingly, AAV vector transduction efficiency in cultured cells may not always be translated into that of animals. For instance, AAV8 induces much higher transgene expression than other serotypes in the mouse liver [5], but not in cultured cell lines.

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Of the 12 serotypes, several AAV serotypes and their variants have been used in clinical trials [6–8]. As the first characterized capsid, AAV2 has been most widely used in gene delivery, such as RPE 65 for Leber congenital amaurosis and Factor IX (FIX) for hemophilia B [8–10]. Although the application of AAV vectors has been proven safe and a therapeutic effect has been achieved in these clinical trials, one of the major challenges of the AAV vector is its low infectivity, and that it requires relatively large numbers of viral genomes [11,12]. AAV8 is another vector that has been used in several clinical trials for patients with hemophilia B. The results from AAV8/FIX liver-targeted delivery have demonstrated that there are distinct species-specific differences in transgene expression between mice, non-human primates, and humans [13–15]. While 10^{10} vector genome (vg) of AAV8 with FIX gene could reach supra-physiologic levels (> 100%) of FIX expression in FIX knock-out mice [15], only high doses (2×10^{12} vg/kg of body weight) could induce detectable FIX expression in humans [10,16]. Based on the results described above, the development of effective strategies to enhance AAV transduction is still necessary. The majority of people have been naturally exposed to AAVs, and as a result, a large portion of the population has neutralizing antibodies (Nabs) in their blood and other bodily fluids [17,18]. The presence of Nabs poses another major challenge for broader AAV applications in future clinical trials [18,19]. Many approaches have been explored to enhance AAV transduction or to evade Nab activity, especially the genetic modification of the AAV capsid based on rational design and directed evolution [20–24]. Although several AAV mutants have demonstrated high transduction, along with the capacity to escape Nab, *in vitro* or in animal models the modification of the capsid can potentially result in a different cell tropism than that of the parental AAVs [25].

Our original studies demonstrated the concept that the capsids from different AAV serotypes (AAV1 to AAV5) were compatible for assembly when contributed from separate AAV serotype capsids [26]. Most available AAV monoclonal antibodies have been characterized at the atomic level and recognize several sites located on different AAV subunits [27–31]. Additionally, recent studies utilizing chimeric AAV capsids have demonstrated that higher transduction can be achieved by swapping a structural domain for a primary receptor or for a tissue-specific motif from different serotypes by classic recombinogenic techniques. For example, the introduction of an AAV9 glycan receptor into an AAV2 capsid enhances AAV2 transduction [32], or substitution of a 100 aa domain from AAV6 into an AAV2 capsid increases muscle tropism [21]. While usually successful, these approaches are dependent on structural analysis knowledge and genetically engineered substrates, which may be time consuming and unpredictable in nature with respect to their final product. Based on these genetically altered AAV capsid genomes, we hypothesize that a polyploid AAV vector might induce a higher transduction efficiency without eliminating the tropism from the parental vectors. A polyploidy AAV vector is defined as a vector which is produced from the co-transfection of capsids from different serotypes parents, or mutant serotype parents that results in a wild-type AAV virion assembled from 60 intact capsomere subunits. Moreover, these polyploid capsids might have the ability to escape Nab since the majority of Nabs recognize conformational epitopes, and the polyploid virions would have subtle changes in their surface structure that might potentially alter such epitopes.

2. Materials and methods

2.1. Cell lines

HEK293 cells, Huh7 cells and C2C12 cells were maintained at 37 °C in 5% CO_2 in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum and 1% penicillin–streptomycin.

2.2. Recombinant AAV virus production

Recombinant AAV was produced by a triple-plasmid transfection system [33]. A 15 cm dish of HEK293 cells was transfected with 9 μg of AAV transgene plasmid pTR/CBA-Luc, 12 μg of AAV helper plasmid containing AAV Rep and Cap genes, and 15 μg of Ad helper plasmid pXX6-80. To generate haploid AAV2/8 virions, AAV2 or AAV8 helper plasmids were co-transfected at three different ratios of 1:1, 1:3 and 3:1. To make triploid AAV2/8/9 vectors, the ratio of helper plasmid for each serotype was 1:1:1. Sixty hours post-transfection, HEK293 cells were collected and lysed. Supernatant was subjected to CsCl gradient ultra-centrifugation. Virus titer was determined by quantitative PCR using a pair of primers that were designed to bind to a homologous sequence on the ITR region. The qPCR assay was conducted using SYBR Green reagents and Light cycler 480 from Roche.

2.3. In vitro transduction assay

Huh7 and C2C12 cells were transduced by recombinant viruses with 1×10^4 vg/cell in a flat-bottom, 24-well plate. Forty-eight hours later, cells were harvested and evaluated by a luciferase assay system (Promega, Madison, WI).

2.4. Animal study

Animal experiments performed in this study were conducted with C57BL/6 mice and FIX −/− mice. The mice were maintained in accordance to NIH guidelines, as approved by the UNC Institutional Animal Care and Use Committee (IACUC). Six-week-old female C57BL/6 mice were injected with 3×10^{10} vg of recombinant viruses *via* retro-orbital injection. Luciferase expression was imaged 1 week post-injection using a Xenogen IVIS Lumina (Caliper Lifesciences, Waltham, MA) following intraperitoneal injection of D-luciferin substrate (Nanolight Pinetop, AZ). Bioluminescent images were analyzed using Living Image (PerkinElmer, Waltham, MA). For muscle transduction, 1×10^{10} particles of AAV/Luc were injected into the gastrocnemius of 6-week-old C57BL/6 females. Mice were imaged at the indicated time points.

FIX knockout male mice (FIX KO mice) received 1×10^{10} vg *via* tail vein injection. This delivery method has similar liver transduction and safety profile to retro-orbital injection (data not shown). At various time points after injection, blood was collected from the retro-orbital plexus. At week 6, mouse bleeding analysis was performed.

2.5. Quantitation of luciferase expression in the liver

Animals utilized for imaging studies were sacrificed at week 4 after recombinant virus injection and the livers were collected. Livers were minced and homogenized in passive lysis buffer. After the liver lysates were centrifuged, luciferase activity in supernatant was detected. Total protein concentration in tissue lysates were measured using the Bradford assay (BioRad, Hercules, CA).

2.6. Detection of AAV genome copy number in the liver

The minced livers were treated with Protease K and total genomic DNA was isolated by the PureLink Genomic DNA mini Kit (Invitrogen, Carlsbad, CA). The luciferase gene was detected by qPCR assay. The mouse lamin gene served as an internal control.

2.7. Human FIX expression, function and tail-bleeding time assays

To determine human FIX expression, one-stage hFIX activity and tail-bleeding time assays were performed as previously described [34,35].