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Reengineered AAV Vectors: Old Dog, New Tricks

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Abstract

Adeno-Associated Viral (AAV) vectors have emerged in recent years as powerful tools for therapeutic gene transfer. Successes in clinical trials and the discovery of several hundreds of naturally occurring AAV isolates have triggered efforts to understand and manipulate this deceptively simple parvovirus for a myriad of gene therapy applications. Exciting breakthroughs based on directed evolution of novel tissue-specific variants from combinatorial AAV libraries have been reported. Recent approaches driven by the availability of structural information have yielded a new generation of reengineered AAV vectors.

Introduction

The past decade has witnessed the explosive growth of Adeno-Associated Viral (AAV) vectors as lead candidates for therapeutic gene transfer. In addition to recent successes in clinical trials (Herzog *et al*., 2010), the excitement surrounding AAV within the gene therapy community can be attributed to the discovery of several hundreds of naturally occurring AAV variants isolated from different species (Vandenberghe *et al*., 2009a & 2009b). These new findings have catalyzed worldwide efforts to understand the structure, infectious pathways, tissue tropisms, mechanisms of persistence and epidemiology of AAV isolates. As the biology of AAV continues to unravel, it is becoming increasingly apparent that despite its lack of pathogenicity, this helper-dependent parvovirus is quite complex.

Much of the diversity amongst different AAV isolates can be traced to the major capsid protein (VP3). The VP3 monomer undergoes self-assembly to form icosahedral virion shells that can be resolved into two-fold (dimer), three-fold (trimer) and five-fold (pentamer) axes of symmetry (Xie *et al*., 2002). Differences in the amino acid sequence of the VP3 subunit of different AAV isolates are primarily located within variable loop regions on the capsid surface. Variations in capsid surface topology, in turn, enable the observed diversity in receptor usage, antigenicity, tissue tropism and host specificity of AAV variants and serotypes (Wu *et al*., 2006). It is within this framework that the complexity of AAV begins to emerge.

For instance, initial cell surface binding of AAV capsids is mediated through glycosaminoglycans. AAV2 has been shown to utilize heparan sulfate as a primary receptor, while AAV1, 4, 5, and 6 appear to utilize sialic acid with different linkage specificities for cell surface binding. Subsequent cellular uptake is thought to be mediated by interaction of AAV capsids with different secondary/co-receptors including integrins αVβ5/α5β1 (AAV2), FGFR1 (AAV2), HGF receptor (AAV2), PDGFR (AAV5) and the Laminin receptor (AAV2,3,8,9).

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Primary and secondary receptors utilized by other AAV serotypes are unknown (Wu *et al*., 2006).

Significant diversity is also observed in the tissue tropisms and gene transfer efficiencies of AAV serotypes 1 through 9 and several isolates (rh.8, rh.10, rh.32.33, rh.64R1) (Wang *et al*., 2010). Notably, upon intravenous (i.v.) administration, AAV serotypes 6, 7, 8 and 9 transduce the heart and liver with high efficiency. In addition, AAV4, 6 and 9 demonstrate moderate transduction efficiency in the lung (Zincarelli *et al*., 2008). Upon i.v. administration, AAV capsids are expected to distribute within the vasculature in different organs and peripheral tissues. Certain AAV serotypes, in particular, AAV6 through 9, appear capable of traversing the vascular endothelial barrier to transduce underlying skeletal muscle tissue. Intravascular injection of AAV9 vectors under certain conditions has also been shown to mediate robust transduction of different cell types within the CNS (Foust *et al*., 2009). Whether similar tissue tropisms are maintained across different species as well as in clinical gene transfer studies are currently being investigated by several research groups.

Rationale

Physiological considerations including transvascular transport, renal and hepatic clearance are pertinent towards understanding the tissue tropism profiles of AAV vectors. The icosahedral AAV capsid has a diameter of ~25nm. It is likely that the hydrodynamic diameter of the virion shell following adsorption of serum proteins in the blood circulation is significantly larger. Whether these serotypes permeate the blood vessel barrier through a transcellular/paracellular pathway or by gradual equilibration between the vascular compartment and the extravascular space through capillary fenestrae remains to be determined. Further, since renal clearance of nanoparticles by glomerular filtration is unlikely, hepatic sequestration is thought to be the major clearance mechanism for viruses and other nanoparticles alike (Di Paolo *et al*., 2009; Schipper *et al*., 2009). Not surprisingly, all naturally occurring AAV serotypes and variants studied to date exhibit a ubiquitous propensity to sequester within the liver, albeit with varying efficiency (Zincarelli *et* al., 2008; Wang *et al*., 2010).

Consequently, development of strategies to redirect AAV vectors from liver to other organs would be tremendously useful from a safety and efficacy standpoint for certain gene therapy applications. For instance, musculoskeletal complications such as muscle weakness, damage and degeneration are seen in case of the muscular dystrophies, neuromuscular diseases such as spinal muscular atrophy and congenital myasthenia gravis as well as lysosomal storage disorders such as Pompe disease (Deconinck and Dan, 2007; Pastores, 2008). Corrective gene replacement is a promising therapeutic strategy for the treatment of such diseases. However, a significant hurdle towards achieving efficient gene transfer in skeletal muscle is the paucity of vectors that can selectively transduce a broad spectrum of muscle groups following systemic administration.

Capsid Reengineering

Unique challenges, such as those outlined above, have triggered efforts to engineer novel, lab-derived AAV strains with disease-specific applications in mind. Several exciting breakthroughs involving the directed evolution of tissue-specific AAV vectors from combinatorial AAV capsid libraries have been reported (Excoffon *et al*., 2009; Li *et al*., 2009; Yang *et al*., 2009). While the latter approach requires minimal information pertaining to AAV capsid structure, “reengineering” existing AAV capsid templates hinges on prior knowledge of capsid surface topology and receptor usage. For instance, insertion of random peptide sequences within variable surface loops of the AAV2 capsid has resulted in the generation of AAV display peptide libraries. *In vivo* biopanning of such randomized viral libraries has yielded novel AAV vectors with distinct tissue-selective peptide sequence motifs.

Although this strategy did not result in tissue-specific gene transfer, such AAV vectors are potentially useful tools for applications warranting expanded tissue tropism (Michelfelder *et al*., 2009). More recently, tyrosine-mutant AAV vectors displaying higher transduction efficiency than parental serotypes were generated after structural analysis of surface-exposed tyrosine residues on the AAV capsid (Zhong *et al*., 2008). Mutation of tyrosine residues to phenylalanine is thought to prevent phosphorylation of AAV capsids, which in turn might help evade ubiquitination and proteasomal degradation. Another example of reengineered AAV vectors is AAV2.5, a lab-derived AAV strain generated by mutagenesis of five surface residues at the two-fold (VP3 dimer) interface of the AAV2 capsid (Bowles and Samulski, unpublished). This reengineered strain was recently tested for intramuscular administration in a Phase I clinical safety trial for Duchenne muscular dystrophy.

Evolution of new viral strains with altered tissue tropism or host specificity in nature is mediated by accumulation of amino acid substitutions that alter receptor specificity and/or binding avidity (Hensley *et al*., 2009). We recently adapted a similar approach to generate a panel of synthetic AAV strains by reengineering a previously identified heparan sulfate receptor footprint on AAV2 (Asokan *et al*., 2010). This effort was made possible due to availability of structural data on the AAV8 capsid as well as the AAV2 capsid-heparin complex (Nam *et* al., 2007; Levy *et al*., 2009). Briefly, the heparan sulfate footprint residue consists of a continuous basic patch of amino acid residues including R484, R487, K527, K532, R585 and R588. The latter two residues (R585 and R588) are located within a variable surface loop and form the inner walls of the spikes located on the icosahedral threefold axis. The other basic residues within the footprint occupy the floor surrounding the spike regions. Earlier studies have demonstrated the potential for abrogating liver sequestration of AAV2 vectors by mutagenesis of basic residues involved in heparin binding (Kern *et al*., 2003).

Our approach yielded several new AAV2-derived strains displaying systemic transduction profile(s) following i.v. administration in mice. One such strain, AAV2i8, contains six amino acid substitutions replacing key heparan sulfate binding residues (R585, R588) and flanking residues with corresponding amino acids from the AAV8 capsid. The synthetic AAV2i8 strain readily traverses the vasculature and selectively transduces cardiac and whole body skeletal muscle tissues with high efficiency. Moreover, unlike other naturally occurring AAV, which are preferentially sequestered by the liver, AAV2i8 vector displayed markedly reduced hepatic tropism. It is interesting to note that mutagenesis studies with AAV6 corroborate the notion that attenuated heparin binding results in decreased liver tropism. The exact nature of capsid-receptor interactions that mediate the shift in tropism from liver to muscle tissue remains to be determined.

Mechanistic Considerations

How does the reengineered AAV2i8 strain traverse the vasculature into peripheral tissue space? Two critical observations comparing AAV2i8 with AAV serotypes 8 and 9 might hold the key towards unlocking the answer to this question. First, AAV2i8 capsids display prolonged circulation time in blood compared to AAV8 and AAV9 vectors. Secondly, a relatively slow onset of gene expression is seen in case of AAV2i8 vectors in contrast to AAV8 and AAV9 vectors, both of which display widespread and rapid onset gene expression. Taken together, the latter findings might support the notion that AAV2i8 vectors gradually equilibrate between the vascular compartment and peripheral tissue by traversing through capillary fenestrae.

Observations from isolated limb infusion studies might provide further evidence for such a transport mechanism. Briefly, this experiment involves isolation of the mouse hind limb using a tourniquet to prevent exchange of vascular contents with the systemic circulation. Subsequently AAV vectors were administered through the saphenous vein at low or high

volumes of injection to assess the effect of such on gene transfer efficiency in hind limb skeletal muscle. Interestingly, AAV8 vectors, but not AAV2i8 vectors appear to benefit from higher injection volumes. No difference in the transduction efficiency is noted between AAV8 and AAV2i8 vectors at low volume of injection. These results suggest that opening of capillary fenestrae at high volumes of injection provides an additional route for transvascular transport of AAV8, but not AAV2i8 vectors.

Clinical Translation

From a translational perspective, AAV2i8 and other such vectors are particularly attractive candidates for therapeutic gene transfer in a broad spectrum of musculoskeletal disorders, which warrant transduction of a wide range of muscle groups following systemic administration. First, the selective muscle tropism of AAV2i8 vectors, when combined with transcriptional targeting strategies such as the incorporation of muscle-specific promoters and miRNA target sites within the vector genome cassette, should allow exquisite control over the transgene expression profile. Secondly, the low level of sequestration within the liver, a likely off-target organ in treatment of musculoskeletal disorders, enables significant improvement in safety profile. The prolonged blood circulation time and long term gene expression profile of AAV2i8 vectors could potentially eliminate the need for repeat vector administration.

Lastly, based on their unique antigenic profile, reengineered vectors appear to be promising candidates for clinical studies. Natural exposure to AAV serotypes and variants has been shown to result in production of capsid-specific antibodies in the human population. The seroprevalence of neutralizing antibodies (NAb) against AAV2 and AAV1 capsids is significantly higher than that of AAV8 and AAV9 (Calcedo *et al*., 2009; Boutin *et al*., 2010). Our studies demonstrate that reengineering the receptor footprint on the AAV capsid surface also alters antigenicity, a key consideration in translational gene therapy studies. The NAb titer against AAV2i8 capsids was found to be low in serum obtained from mice pre-immunized against AAV2 capsids. Further, seroprevalence of NAbs against AAV2i8 in human serum was as low as that associated with AAV8 capsids. This finding is particularly relevant towards gene transfer applications involving systemic administration of AAV2i8 vectors.

Future Potential

As mentioned earlier, capsid reengineering hinges on structural information pertaining to AAV capsids and complexes with their cognate receptors. Crystal structure information for AAV serotypes 2, 4 and 8 are currently available (Xie *et al*., 2002; Govindasamy *et al*., 2006; Nam *et al*., 2007). Preliminary X-ray structural studies of AAV1, 5, 6, 7 and 9 have been carried out (DiMattia *et* al., 2005; Miller *et* al., 2006; Quesada *et* al., 2007; Xie *et al*., 2008; Mitchell *et al*., 2009). High resolution structures of different AAV capsids complexed with glycans and/or co-receptors derived using X-ray crystallographic analysis, cryo-electron microscopy and three-dimensional image reconstruction will likely be available in the near future. Reengineering various receptor footprints on different AAV strains will provide further insight into the infectious pathways, tissue tropisms and host specificities. Exploration of sequence space within the specific framework of AAV capsid surface domains will also enable the generation of an ever-expanding synthetic AAV toolkit for human gene therapy applications.

In summary, reengineered AAV vectors derived from natural AAV isolates add an important dimension to vector development. The strategy demonstrates tremendous potential to generate new AAV strains with atypical transduction profiles and antigenicity suitable for translational gene transfer studies. The subsequent availability of organ-selective AAV vectors (or those selectively detargeted from off-target organs) will provide the clinical community with reagents that facilitate disease-specific treatment strategies for human gene therapy.

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