VECTOROLOGY OF ADENO-ASSOCIATED VIRUSES (AAV)*

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Abstract
In vivo gene replacement is one of the most compelling concepts in modern medicine. Adeno-associated virus (AAV) vectors are currently among the most frequently used viral vectors for gene therapy and they have shown therapeutic efficacy in a range of animal models. The lack of pathogenicity of the virus, low immunogenicity, its stability, and many available serotypes have increased AAV's potential as a delivery vehicle for gene therapy applications. There are some limitations to the use of rAAV in gene therapy. The first is their size. Due to the small size of the vector, the ability to conduct a therapeutic gene expression cassette is limited. Another limitation is the common occurrence of neutralizing antibodies in human populations. This review will focus on the biology of AAV, its use as a vector for gene therapy and mechanisms of AAV/host cell interaction.

Key words: Adeno-Associated Virus, AAV, gene therapy, neutralizing antibody

INTRODUCTION
Adeno-associated virus-based vectors are currently used in clinical trials for the gene therapy of many diseases, such as cystic fibrosis, muscular dystrophy, Batten’s disease, Parkinson’s disease, Leber’s congenital amaurosis and hemophilia B (1). The first drug approved in the United States and Europe based on rAAV (recombinant adeno-associated virus), called Glybera, has been launched. RAAV-based vectors are used in gene therapy because of its many advantages and low number of disadvantages.

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The main advantage of AAV-based vectors are: no pathogenicity, the ability to infect both dividing and non-dividing cells, low immune response from the host and the ability to deliver genes into different tissues (many different serotypes). The disadvantage of using them is their small size, which limits the size of the therapeutic gene, and the presence of the neutralizing antibody in human serum which may limit the administration of the vector.

Adeno-associated viruses (AAV) were discovered in electron micrographs as contamination in adenovirus preparations and soon became the subject of interest of scientists around the world. In the early 90s in 1984 Hermonat and Muzycka (2) published the first paper on cloning of the AAV vector which was capable of expressing foreign genes in mammalian cells. Thirty years have passed since that time and the interest in the subject of AAV as a vector in gene therapy continues to grow.

It is estimated that 90% of the human population is AAV seropositive. However, these viruses do not cause any known disease in humans (3). The AAV virus belongs to the parvovirus family, specifically the dependovirus genus. Classification of the parvovirus family is based on virus size, as they are the smallest of all known viruses. In addition, the members of the dependovirus genus require a helper virus, such as the adenovirus or herpes simplex virus, to facilitate productive infection and replication.

**AAV VIRUS**

The best known representative of all the AAV viruses is serotype 2: AAV-2. AAV-2 is very small, as the AAV capsid is approximately 22 nm. It is an icosahedral and nonenveloped virus. AAV particles contain a single stranded DNA genome consisting of approximately 4.7 kb that could be divided into three functional regions: two open reading frames (ORFs, rep and cap) and inverted terminal repeats (ITRs). (4). The ORFs are flanked by inverted terminal repeats. These 145-nucleotide ITRs are partially paired and they fold upon themselves to maximize base pairing and form a T-shaped hairpin structure. The first 125 nucleotides of the ITR constitute a palindrome, whereas the other 20 bases (called the “D” sequence) remain unpaired. The key role of the ITRs consists in AAV DNA replication. Located on the ITRs are the Rep protein binding element (RBE) and terminal resolution site (TRS), which are critical for the replication process. In the current model of AAV replication, the ITR is the origin of replication and serves as a primer for second-strand synthesis by DNA polymerase. In addition, this sequence is also essential for AAV genome packaging and site-specific integration into the host genome. Between the ITR sequences there are two open reading frames (ORFs) containing the Rep and Cap gene. The Rep gene produces four Rep proteins, Rep78, Rep68, Rep52, and Rep40. These proteins are nonstructural (functional) proteins that are involved in the viral genome replication, transcription control, and packaging of DNA into new capsids. (5). The Rep gene contains two viral promoters known as P5 and P19, which regulate the transcription of the four Rep proteins with apparent molecular masses of 78, 68, 52, and 40 kDa (Rep78 and its splice variant Rep68, and Rep52, as well as its splice variant Rep40, respectively). The larger Rep proteins (Rep78 and Rep68) are important regulatory proteins which positively (in the presence of the helper virus) or negatively (in the absence of the helper virus) regulate AAV gene expression, and are required for DNA replication (6). The smaller Rep proteins, Rep52 and Rep40, are involved in the accumulation of viral DNA used for packaging within AAV particles. All Rep proteins possess helicase and ATPase activity. In addition, the Rep 78 and 68 proteins possess strand and site-specific endonuclease activity (nicking at the TRS) and site-specific DNA binding activity (binding at the RBE) (6). The cap gene produces three viral capsid proteins known as VP1, VP2, and VP3 using the P40 promoter. The molecular ratio of these proteins (VP1:VP2:VP3) are present in a 1:1:10 ratio. The VP1 protein (87 kDa) is the biggest of the capsid proteins and it has been implicated in the infectivity of the virus. The structural protein VP3 is the smallest protein and is probably responsible for tissue tropism. The VP2 protein (72 kDa) seems to be necessary for capsid assembly (7). Differences in the amino-acid structure of the capsid protein VP1, VP2, VP3 are the basis for distinguishing at least 12 serotypes of AAV. Each serotype of AAV uses a different receptor on the cell surface to enter the cells, leading to the many different tissue tropisms of the various serotypes. The studies so far have shown that AAV vectors efficiently infect such cells as: the skeletal muscle (AAV1, 8,9), cardiac (AAV9), liver (AAV8), central nervous system (AAV5), kidney (AAV2) and sense organ cells (retinal pigment epithelium AAV4 and 5 cochlea hair cells AAV3) (8).

Detailed information about AAV serotypes were published in the *Progress of Biochemistry* by one of the co-authors of this article (9).

**IMMUNE RESPONSES TO AAV VECTORS**

One of the biggest challenges which hinder the use of rAAV in gene therapy is the immune response of the host. In this case the host immune response mainly consists of cellular and humoral responses, while innate immunity is weak (10). The cellular response is mediated by cytotoxic T-cells that have the potential to destroy transduced cells. The humoral response is via a neutralizing antibody (NAb). NAbs commonly circulates in humans after AAV infection and they prevent re-infection. What is interesting according to some authors, is that the humoral response appears to be dependent on T-cells; it was shown that anti-CD4 antibodies inhibited T-cell function and prevented the formation of neutralizing antibodies to AAV, therefore allowing AAV to easily infect the cell (11, 13). However, such correlation was not observed in a large group of healthy donors (12).
Although neutralizing antibodies belong to 4 subclasses of IgG, most of them are IgG1. Recent studies have shown that epitopes of NABs raised against capsids of different AAV serotypes are located on receptor-binding sites and transduction determinants. These epitopes suggest potential mechanisms for virus neutralization by the antibodies (14).

Immune responses against the AAV capsid depend on the target organ, route of administration, AAV serotype and transgene. A strong cellular response was observed after AAVs were administered intraperitoneally, intravenously or subcutaneously, whereas a lower cellular response was observed after intramuscular injection (15). This correlates with the lower tendency of pre-existing neutralizing antibodies to block in vivo gene transfer to the skeletal muscle (17). Humans are a natural host to the AAV virus (18). A recent study concerning the prevalence of neutralizing antibody titers (NAB) to the various AAV serotypes spanning 4 continents has shown that the most prevalent NABs are AAV2 followed by AAV1, while AAV8 and AAV7 have the least prevalent responses (18).

It is also possible that in the serum, in addition to neutralizing antibodies, there are other neutralizing factors (NAF) against wild-type AAV, which may be at least partially responsible for the neutralization of the virus (19).

**RAAV VECTORS – PRODUCTION AND PURIFICATION**

AAV vectors have only about 300 nucleotides of the native viral sequence (ITR site), which improves the safety of gene therapy. Production of the recombinant AAV virus is based on adherent cell lines, such as HEK293 cells. Cells are transfected with appropriate plasmids that contain formation genes essential to the virus, such as rep, cap, helping virus genes and the target gene in the therapy. The cells are then cultivated on 10 - 50 fifteen-centimetre plates. The number of plates depends on the scale of production. For larger-scale production, bioreactors are used.

The target gene should be inserted between ITR sequences and is then packaged into new virus particles. The rep and cap genes, which in the wild type AAV virus are between these sequences, are supplied on a separate plasmid. Previously in the production of rAAV, co-infection with a helper virus (adenovirus) was used. However, this had the potential to generate a contaminating rAAV adenovirus. Now it is known which adenovirus genes are essential to the formation of rAAV, so they are delivered in a separate plasmid (these genes are E1a, E1b, E2a, E4 and VA). There is also the possibility to provide rep and cap genes and adenovirus genes on a single plasmid, which reduces the amount of plasmids necessary for the transfection.

The next step is virus purification. The procedure begins with ultracentrifugation in a cesium chloride gradient or density of iodoxanol. Then in the case of serotype 2, the *heparin affinity column* is used.

**MECHANISM OF CELL INFECTION BY AAV**

AAV is replicated within the host cell and this is completed in several stages: -AAV interaction with the corresponding receptor located in the plasma membrane of target cells, -internalization of the virus, -transport to the nucleus, and DNA AAV synthesis. The adhesion of the virus to the cell membrane is possible by using the cellular receptor heparan sulfate proteoglycan (HSPG). Internalization is enhanced by interactions with at least six known coreceptors, including V5 and V1 integrins, the hepatocyte growth factor receptor, the fibroblast growth factor receptor 1, and the laminin receptor (11). The processes of virus transport into the cell are not completely characterized. It takes place on the basis of endocytosis in clathrin-coated vesicles. Dynamin (a soluble intracellular protein with GTPase activity) is a protein that is necessary at this stage. It has been shown that cells defective in dynamin are much more difficult to infect by AAV (20). Recently, much attention has been paid to the study of the participation of the cells’ cytoskeleton in viral infection. The cytoskeleton is a network inside the cell composed of poorly soluble proteins which include microtubules, actin microfilaments and intermediate filaments. The cytoskeleton is a dynamic structure which is involved in the cell’s movement, intracellular transport and movement of organelles. It is not surprising that the viruses use them during the infection and replication cycle and for transport of progeny virions or their components (22). In the case of the microtubule network, this cytoskeletal component is required for the transport of the virus (contained in endocytic vesicles) near the nucleus (23). This transport is fast and unidirectional in contrast to other viruses in which the transport is bidirectional or slow (23). For AAV transport, the cytoskeleton is not the only way, because the destruction of the microtubule network does not completely block the transduction of the virus but only reduces it by about 2 fold (24).

It seems that the low pH in the late endosomes is essential for virus escape (near the nucleus) and successful infection. In *in vitro* studies using bafilomycin A1 (a drug that inhibits the proton pump for endosomes), it was shown that this drug inhibited the progression of AAV infection (25). Following release from the endodome, the AAV then migrates to the nucleus.

**THE LIFE CYCLE OF AAV AND RAAV**

The life cycle of AAV is dependent on the presence or absence of a helper virus. In the presence of a helper virus (adenovirus or herpesvirus), the AAV lytic cycle proceeds. During this period, AAV undergoes productive infection characterized by genome replication, viral gene expression, and virion production. In the absence of an adenovirus or herpesvirus, there is limited AAV replication, viral gene expression is repressed, and the AAV genome can establish latency by integrating into a 4-kb region on chromosome 19 (q13.4), named AAVS1 (36, 37). The AAVS1 locus is near several muscle-specific genes known as TNNT1 and TNNI3 (26) and contains sequences...
similar to those found in the viral ITR sequences (RBE and TRS) separated by 8 nucleotides (21). Although the human genome contains more sites similar to RBE, only in this position on our genome are the sequences similar to viral RBE located next to a sequence similar to the TRS (28). The life cycle of recombinant AAV vectors, which, in place of the viral rep and cap genes contain the target gene, refers to the introduction of this gene into cells, and their synthesis. RAAV is rather rarely integrated into the host and this integration is in a random location because of the lack of the Rep 78 protein which is important in this process (27). This is an advantage of using AAV, because it reduces the risk of neoplastic transformation after using them.

**SUMMARY**

Recombinant AAV vectors are used in gene therapy in the clinic. They are efficient in introducing genes into normal and malignant cells. Further development of AAV vectors should make it possible to obtain pure, pharmacopoeial preparations with a high titre rAAV targeting of selected types of cells, tissues and organs.

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