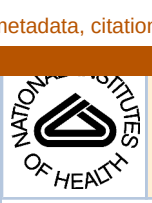

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Adeno-associated virus vectors: potential applications for cancer gene therapy

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Abstract

Augmenting cancer treatment by protein and gene delivery continues to gain momentum based on success in animal models. The primary hurdle of fully exploiting the arsenal of molecular targets and therapeutic transgenes continues to be efficient delivery. Vectors based on adeno-associated virus (AAV) are of particular interest as they are capable of inducing transgene expression in a broad range of tissues for a relatively long time without stimulation of a cell-mediated immune response. Perhaps the most important attribute of AAV vectors is their safety profile in phase I clinical trials ranging from CF to Parkinson's disease. The utility of AAV vectors as a gene delivery agent in cancer therapy is showing promise in preclinical studies. In this review, we will focus on the basic biology of AAV as well as recent progress in the use of this vector in cancer gene therapy.

Keywords

adeno-associated virus vector

Gene therapy was initially designed for treatment of genetic diseases. To date more than 400 gene therapy clinical trials have been conducted worldwide, over half of them related to cancer therapy. One attractive viral vector for gene therapy is adeno-associated virus (AAV). Although there have been fewer studies examining AAV vectors for cancer gene therapy than other viral vectors, numerous advantages of AAV make it an ideal vehicle for the delivery of genes. These desirable traits include its abilities to infect both dividing and nondividing cells, to transduce a broad range of tissues *in vivo* such as brain, liver, muscle, lung, retina, and cardiac muscle, and to initiate long-term gene expression in these tissues. Furthermore, wild-type AAV does not cause any known disease and does not stimulate a cell-mediated immune response.[1] Among the serotypes of AAV, only AAV-2 vectors are now being used for clinical gene transfer for cystic fibrosis, hemophilia, and Canavan's disease.[2] Here, we review the biology of AAV, recent progress on recombinant AAV vectors (rAAV), and the status of rAAV as cancer gene therapy vectors.

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Biology of AAV

AAV is a single-stranded DNA parvovirus with a genome of 4700 nucleotides. As a member of the dependovirus subfamily, AAV is reliant on another virus such as adenovirus or herpes simplex virus to complete its life cycle. In the absence of this helper virus, AAV can establish latency by integrating site specifically into the human chromosome 19 AAVS1 site.[3]

The genome of AAV contains two open reading frames (ORF) and palindromic inverted terminal repeat elements (ITR) that flank the two ends of the genome. These ITR are the minimal *cis*-acting elements necessary for the integration and/or rescue of the AAV genome during the latent stage of its life cycle as well as for the replication of the viral genome and its subsequent packaging into the capsid shell. In addition, only these 145 base ITRs are required in *cis* to generate recombinant AAV; all other viral sequences are supplied in *trans*.[4]

The proteins responsible for replication (rep) are encoded by the left ORF, while the right ORF encodes for the structural proteins of the capsid (Cap). The three capsid proteins (Vp1, Vp2, and Vp3) are generated from a single gene from which translation is initiated at different start codons. As a result, these structural proteins have identical C-termini, but possess unique N-termini. The Cap proteins differ in both size and their distribution in the capsid. Vp1 is the largest with a molecular weight of 87 kDa, while Vp2 and Vp3 have molecular weights of 73 and 62 kDa, respectively.[5] Vp3 is the most abundant protein comprising nearly 80% of the total protein in intact capsids, while Vp1 and Vp2 each represent 10%.[4,6,7]

AAV infection involves a multistep process beginning with virus binding to the cell surface, followed by viral uptake, intracellular trafficking, nuclear localization, uncoating, and second-strand DNA synthesis.[8–13] AAV2 initiates infection by binding to its primary receptor, heparan sulfate proteoglycans (HSPG).[8] Recent work has mapped the heparin-binding site in the AAV2 capsid to two amino acids (a.a. 585 and 588) which appear to play a significant role in binding HSPG.[14,15] $\alpha_v\beta_5$ integrin and fibroblast growth factor receptor 1 (FGFR1) have been recognized as coreceptors for AAV2 infection.[9–11] FGFR1 probably functions to enhance the viral attachment process,[10,11] while the role of $\alpha_v\beta_5$ integrin may involve viral endocytosis, mediated mainly by clathrin-coated pits.[11,13,16] In addition, AAV2 binding to the cell surface via $\alpha_v\beta_5$ integrin may activate Rac1, which induces the stimulation of phosphoinositol-3 kinase (PI3K), culminating in the rearrangements of microfilaments and microtubules that support trafficking of AAV2 to the nucleus after endocytosis.[16]

In order for AAV to continue its lifecycle, it must be released from the endosome. Low endosomal pH appears to be necessary for this,[11,17] and the phospholipase A2 (PLA2) domain located in the VP1 unique region may also play a role.[18] Following escape from the endosome, AAV rapidly travels to the cell nucleus and accumulates perinuclearly beginning within 30 minutes after the onset of endocytosis. Within 2 hours, viral particles can be detected in the cell nucleus, suggesting that the AAV particle enters the nucleus prior to uncoating.[11,19] Interestingly, the majority of the intracellular virus remains in a stable perinuclear compartment.[20,21] Perinuclear accumulation appears to be problematic for both permissive and nonpermissive cells, suggesting that the process of nuclear entry may also affect vector transduction in certain cell types.[22]

After receptor binding, internalization, and nuclear entry, AAV virions uncoat and release a single-stranded DNA template, which must be converted to a duplex intermediate before transcription can ensue. The efficiency of forming the complementary strand can significantly impact vector transduction.[23,24] Defects in any or all of the stages of viral infection discussed above can influence the resulting transduction profiles of recombinant AAV in different cell types.

AAV serotypes

Until recently, the majority of the research conducted using AAV-based vectors employed serotype 2. Vectors based on AAV2 have been the most studied and are currently used in clinical trials for some inherited diseases.[2,25–28] Preliminary data from these patients has further emphasized the safety of AAV for human applications. Stable and efficient transgene delivery by AAV2 has been demonstrated to correct diseases in animal models. For example, AAV2 vectors delivering genes encoding antiangiogeneic proteins, neurotrophic factors, or proteins with retinal specific functions have shown promise for the rescue of retinal degeneration in various animal models.[29–37] Attempts to correct the factor IX deficiency of hemophilia B by delivery of an AAV2 vector encoding FIX to muscle, liver, and lung in different animal models resulted in sustained FIX expression lasting for the lifetime of the animal.[38–45]

To date eight additional primate serotypes of AAV have been identified,[46–52] the majority of which have been isolated as contaminants of adenoviral cultures. AAV5, however, was isolated from a condylomatous lesion,[53] while both AAV7 and 8 were cloned from rhesus monkey tissues.[48] With the exception of AAV6, which differs from AAV1 by only six amino acids, all serotypes show a significantly different amino-acid sequence in the capsid proteins.[48,54] Neutralizing antibody assays have revealed complete crossneutralizing antibody response between AAV1 and AAV6, and only partial crossreaction between AAV2 and AAV3 (Li and Samulski, unpublished data).[48,52] No evidence of crossreacting neutralizing antibody was seen between any of the other serotypes. Recently, the receptors for other serotypes have been identified. AAV-4 and AAV-5 use α2–3 *O*-linked and N-linked sialic acid for cell binding, respectively,[55] and the PDGF receptor was also found to play a critical role in AAV5 infection.[56] The cellular receptors for serotypes 1, 6–9 have yet to be determined.

Many *in vivo* studies have clearly demonstrated that the various AAV serotypes display different tissue or cell tropisms. AAV-1 and AAV-7 are the serotypes most efficient for the transduction of skeletal muscle.[48,51,57] AAV-3 is superior for the transduction of megakaryocytes.[58] AAV-5 and AAV-6 infect apical airway cells efficiently.[59,60] AAV-2, AAV-4, and AAV-5 transduce different types of cells in the central nervous system.[61] AAV-8 and AAV-5 can transduce liver cells better than AAV-2.[48,62] AAV-4 was found to transduce rat retina most efficiently, followed by AAV-5 and AAV-1.[63,64] Further work on AAV serotypes should result in the identification of all domains involved in receptor binding and trafficking. This information will be useful in the development of AAV retargeting vectors.

By using different AAV serotypes in muscle and liver, higher gene expression levels of FIX were observed than with AAV2 FIX vectors.[65,66] The tropism of AAV serotypes has been tested in the CNS, and disease phenotype has been corrected or improved in the animal models of brain disorders, such as Parkinson's disease,[61,67–73] lysosomal storage diseases,[74,75] or seizures.[76,77] In addition, AAV efficiently transduces the rodent heart suggesting that it may be useful to treat heart diseases.[78–84]

Recent progress in AAV vector development

The expansion of the field of AAV-based gene therapy has been driven by continuing research of the biology of this unique parvovirus.[4,85,86] Here, we will review progress in improving gene delivery by modification of the capsid as well as strategies employed to increase transgene expression.

Modification of AAV capsid to change tropism

The tropism of AAV has been limited to particular cell types but can be expanded to include other cell types through modification of the capsid to target specific cells or enhance AAV transduction. Three approaches have been used to modify AAV virions: receptor targeting, mixed capsids in the shell of the virion, or marker rescue to produce recombinant virus.

Receptor targeting.—Two different strategies have been used to achieve AAV receptor targeting: chemical cross-linked bifunctional antibodies and genetic manipulation of the capsid gene. Two groups[87,88] have exploited the use of bifunctional antibodies to target AAV2 virus to a nonpermissive cell line. Bartlett et al described a bispecific $f(ab'\gamma)_2$ antibody with specificity for the AAV2 capsid and the surface receptor $\alpha_{IIb}\beta_3$ integrin. After incubation of virus with the antibody, the viruses were able to transduce human megakaryoblast cells (DAMI and MO7e),[87] which typically are nonpermissive for AAV2 infection. Ponnazhagan et al used a novel conjugate-based targeting method to enhance tissue-specific transduction of AAV2-based vectors. In this approach, the high-affinity biotin–avidin interaction was utilized as a molecular bridge to crosslink purified targeting ligands. A recombinant bispecific protein containing sequences of human epidermal growth factor (EGF) or human fibroblast growth factor 1α (FGF 1α) as a target cell ligand was genetically fused to core-streptavidin. Conjugation of the bispecific targeting protein to the vector was achieved by biotinylated rAAV-2. The incubation of virus with conjugated protein led to a significant increase of transduction in EGF receptor-positive SKOV3.ip1 cells and FGF receptor 1 alpha-positive M07e cells, respectively.[88] The availability of high-affinity viral surface binding molecules such as monoclonal antibodies makes the above approaches feasible when knowledge of the three-dimensional structure of the viral surface is lacking. Limitations to the bifunctional antibody approach include how stably and efficiently the intermediate molecule interacts with the virus and the binding affinity of the intermediate to cell-specific receptors, which allow virus uptake and correct intracellular trafficking.[89]

A second approach for receptor targeting is to genetically alter the capsid-coding region. Prior to the elucidation of the AAV crystal structure, three strategies have been employed to determine domains of AAV surface that can be modified:[54] (a) sequence alignment of AAV2 and other parvoviruses with known crystal structure,[90,91] (b) randomly insertional mutagenesis of the entire AAV-2 capsid genome,[92–94] and (c) incubation of AAV2 neutralizing serum with AAV2 capsid peptide pools to define the peptides which represent immunogenic regions on the virion surface.[95]

By aligning the capsid sequences of AAV2 and CPV, Girod et al predicted six sites (amino-acid positions 261, 381, 447, 534, 573, 587) that could tolerate the insertion of a targeting ligand. Using the 14 amino-acid peptide L14 that included an RGD motif as a ligand, they were able to generate rAAV with packaging efficiencies similar to wild-type AAV2. Furthermore, one insertion mutant (at position 587) efficiently transduced tumor cell lines that express the L14-specific integrin receptor on the surface.[90] Grifman et al[91] aligned the AAV2 capsid to AAV serotypes 1, 3, 4, and 5 and demonstrated the potential insertion sites that are identical to the findings of Girod et al.

The first attempt to target specific cells was the insertion of a single-chain antibody against human CD34, an antigen found on the surface of hematopoietic progenitor cells, at the N-terminal of VP1, VP2, and VP3.[96] The virions made from the mixture of mutant and wild-type capsids exhibited significantly increased infectivity for the CD34-positive human leukemic cell line KG-1, which is refractory to wild-type rAAV infection. Wu et al[92] used a similar strategy to demonstrate particle production with the insertion of the serpin receptor ligand at the N-terminal of VP2. This ligand-containing virus exhibited 15-fold higher transduction for the lung epithelial cell line IB3 than wild-type AAV2, indicating that the N-

terminus of VP2 is exposed on the surface of the virion similarly to canine parvovirus (CPV).[97,98]

The identification of the regions located on the surface of the capsid and the corresponding amino acids responsible for heparin sulfate binding is instrumental for subsequent genetic modifications to the AAV vector. To this end, both Rabinowitz et al[93] and Wu et al[92] have used a random site-directed mutagenesis approach, which has provided a wealth of information for later structural study of AAV2.

Recently, Reid et al[99] combined the two approaches of chemically crosslinked bifunctional antibodies and genetic manipulation of the capsid gene to change the tropism of AAV2. In this study, a peptide (Z34C) from *Staphylococcus aureus* protein A was inserted at position 587 of the AAV2 capsid. Since protein A recognizes and binds to the Fc fragment and not the Fab domain of immunoglobins, the Fab domain remains free to bind the antigen. rAAV2-Z34C vectors coupled to antibodies against CD29 (beta(1)-integrin), CD117 (c-kit receptor), and CXCR4 specifically transduced human hematopoietic cell lines M-07e, Jurkat, and Mec1, respectively.

Recently, Xie et al[100] determined the atomic structure of AAV-2 by X-ray crystallography. This study showed that the core β-barrel motif of the AAV2 capsid monomer, comprised of two antiparallel β-sheets, is very similar in structure to that of other parvoviruses.[101–104] Major differences between AAV2 and the other parvovirus capsid monomers were noted in the loops between the strands of the core β-barrel. These looped structures correspond to regions of the capsid responsible for interactions with antibodies and cellular receptors. An interesting feature of the AAV-2 surface topology are three clusters of three peaks centered about the three-fold axis of symmetry.[105,106] These peaks arise from the interaction of two neighboring subunits. Recently, five amino acids (arginines 484, 487, 585, and 588 and lysine 532) were identified that mediate the natural affinity of AAV2 for HSPG. These five amino acids contribute to a basic patch on one side of each three-fold related spike in the atomic structure of AAV2.[14,15,100]

Marker rescue.—By systematically exchanging domains between AAV1 and AAV2, Hauck et al have identified regions on the AAV1 capsid responsible for its ability to transduce skeletal muscle. This approach, which relied on cloning and assessing each individual construct, was successful and demonstrated the importance of such a strategy.[107] A marker rescue approach has also been used as an alternate method to generate chimeric AAV vectors. One advantage of this method is that domains are swapped based on selection and function in the specific cells or tissues. Several observations have indicated the feasibility of this approach. First, recombination occurs between the serotypes in nature. For example, AAV6 appears to be the product of recombination between AAV1 and AAV2.[51] Second, as few as six nucleotides of homology between two AAV2 mutant genomes was enough to result in recombination.[108] Third, a high degree of homology exists among capsid sequences of known serotypes, about 80% homology among serotypes 1, 2, 3, 7, and 8, and around 60% homology between serotypes 4 or 5 and the other serotypes.[48,54] This homology is distributed throughout the whole capsid genome and provides the platform for recombination between individual serotypes.

We recently completed a study using AAV type 2 DNA in “marker rescue” experiments with AAV3 capsid DNA. The homology between the capsid DNA of the two serotypes allowed for homologous recombination, resulting in a mixed population of chimeric viral genomes at the transfection stage. The chimeric capsid DNA was PCR amplified, cloned into a shuttle vector, and examined for biological properties. According to the crossover regions, four classes of chimeras were observed. The smallest recombinant only had a 16-nt fragment from AAV3 capsid, whereas the largest one contained the whole type 3 capsid sequence that was used for

"marker rescue".[109] Marker rescue is a very valuable technique to map or define the domains that play a role in the AAV transduction process, including receptor binding, trafficking, and uncoating in specific cells. Most importantly, a panel of chimeric viruses generated from marker rescue in specific cells will exhibit novel tropisms and high transduction efficiency for vector targeting in human gene therapy.

Mixed capsid vector.—Based on the high degree of homology between the amino-acid sequences of the different AAV serotypes and what is known about the AAV2 crystal structure, it is possible to form a virion shell from capsid subunits of different serotypes to generate mixed capsid vectors. For example, each virion from mixed vectors of AAV1 and AAV2 should have six kinds of capsid proteins: AAV1 Vp1, Vp2, Vp3 and AAV2 Vp1, Vp2, Vp3. These types of mosaic virions may exhibit a broader tissue tropism due to the combination of the tropisms from different serotypes, or they may exhibit enhanced transgene expression since different serotypes may have different cellular trafficking pathways that serve to initiate transgene expression more efficiently. They may be more readily purified using a heparin column or sialic acid column if the mosaic virions contain capsids from AAV2 or AAV4/5. Additionally, they may be more stable and therefore more readily produced in high amounts than vectors made by epitope insertions into the capsid.[90,92,93,96,110,111]

Hauck et al mixed AAV1 and AAV2 capsids to produce AAV1/2 mosaic virions. These viruses bound heparin and reacted with neutralizing antibodies against both AAV1 and AAV2. When injected into mouse muscle and liver, the AAV1/2 mosaic virus composed of 50% AAV1 and 50% AAV2 induced higher transgene expression than AAV1 or AAV2 vectors alone.[110] We have extended these studies to assess the mixing of serotypes 1–5 and based on data from these experiments propose that three subgroups of AAV exist according to their abilities to generate mixed capsids.[111] Similar mixing experiments performed with newly identified AAV serotypes should allow assignment of these newer serotypes to an appropriate subgroup and may reveal new and novel properties.

Split vectors

The optimal packaging size of rAAV vectors is between 4.1 and 4.9 kb with a maximum capacity of 5.2 kb.[112,113] This small packaging size of AAV has always been thought to preclude its use for delivering genes larger than 5 kb, such as dystrophin and factor VIII, or the use of large regulatory elements to enhance or control transgene expression. Recently, a new approach has been developed to overcome this vector size limitation by exploiting the unique heterodimerization ability of AAV DNA.[114–117] rAAV genomes often form head-to-tail concatemers through intermolecular recombination.[118,119] Therefore, by splitting a gene and its regulatory elements into two separate rAAV vectors, head-to-tail heterodimers of the two rAAV vectors will be formed after codelivering two vectors into target cells. The presence of an appropriate intron or splicing signal sequences then allows rejoining on an intact expression cassette following post-transcriptional processing.[120] This split-gene or trans-splicing strategy has effectively increased the packaging capacity of rAAV vectors to 10 kb and has been applied to factor VIII (F8) cDNA (7 kb). After intraportal vein injection of two rAAV/F8 vectors in immunodeficient mice, 2% of the normal level of factor VIII was achieved for 4 months.[121] The major disadvantage of this approach is the reduced transgene expression due to low recombination of the few genome copies in the nucleus. However, studying the mechanism of AAV concatemer formation should provide novel approaches to improve this type of recombination and enhance transgene expression. In addition, the use of other serotypes may increase recombination because certain serotypes may transduce more efficiently and introduce more copies of the AAV genome to the nucleus.[122]

Self-complementary vectors

The second-strand synthesis is a limiting factor for rAAV transduction.[23,24] Early studies demonstrated that DNA of less than half the size of the wtAAV genome can be packaged as a dimer or a diploid monomer.[4,112,123] Based on this finding, McCarty et al recently generated a novel double-stranded AAV vector that was named self-complementary vector (scAAV). In HeLa cells, scAAV demonstrated an increase in transduction capacity of 5–140-fold over conventional rAAV. Inhibitors of DNA replication did not affect the transduction efficiency of scAAV vectors. Consistent with *in vitro* results, delivery of scAAV/mEpo vectors into mouse liver resulted in much faster and higher transgene expression than the full-length single-stranded DNA vector. This indicates that the scAAV vectors initiate transgene expression immediately after virus uncoating and entry into the nucleus.[124,125] scAAV vectors will be extremely useful when addressing concerns about current vector production and rapid, high transgene expression for AAV vectors in clinical trials.

Potential application of AAV vectors in cancer gene therapy

Although recombinant adenoviral vectors have been utilized for a majority of both preclinical and clinical trials in cancer gene therapy, studies in animal models have suggested therapeutic benefits for tumor treatment using AAV vectors. No T-cell-mediated cytoxicity to AAV vectors has been observed even though AAV vectors can induce strong humoral immune response. AAV can initiate long-term transgene expression and this transduction is attributed to episomal concatamer formation without integration into host chromosome. Based on this point, AAV vectors would appear less mutagenic. Although AAV package capacity is restrained to less than 5 kb, most of therapeutic genes for cancer treatment fall into this range. Some initial limitations of AAV now appear resolved. Production protocols allow for high titer and can be scaled up. Slow onset of gene expression, believed to be related to conversion of ssAAV vector genome to double-stranded templates, now demonstrate fast kinetics when delivered as scAAV vector. This advancement, which further reduces AAV packaging size (2.5 kb), will still accommodate most anticancer genes (e.g. cytokines, RNAi, antiangiogenesis genes, etc.). With new serotypes and potential to develop targeting vectors, AAV holds great promise as a viral vector delivering therapeutic genes such as immune regulation (e.g. cytokines) and antiangiogenesis genes (e.g. endostatin, angiostatin, PEDF) for cancer gene therapy. Cancer gene therapy with AAV vectors is still in its infancy; however, AAV-mediated cancer gene therapy has shown promising results in preliminary experiments. The approaches for cancer gene therapy with rAAV vector can be divided into four major categories: antiangiogenesis, immuno-modulation, suicide gene therapy, and repair of damaged tumor cells.

Antiangiogenesis therapy

Targeting the vasculature has been proven to be an attractive strategy in the treatment of cancer since solid tumor growth and metastasis depend on angiogenesis.[126] To date, AAV2-based vectors have been used to deliver different antiangiogenesis genes to tumors in animal models with promising results.[127–131] Ma et al used an AAV2/angiostatin vector to treat human glioma in an animal model. Following either intratumoral or intramuscular injection with this vector, approximately 40% of the animals survived for over 10 months free of tumors.[127, 129] AAV2/angiostatin vectors delivered directly to the liver via portal vein injection significantly suppressed the growth of established metastatic EL-4 lymphoma tumors in the liver and prolonged the survival time of the animals.[131] In another study, AAV2/angiostatin delivered to the liver via portal vein injection or tail vein injection initiated long-term sustained angiostatin expression in the sera, resulting in inhibited tumor growth in B16F10 melanoma and Lewis Lung Carcinoma (LLC) models within a narrow dose range. The addition of chemotherapy extended the survival of tumor-grafted mice treated with AAV2/angiostatin.[132]

In addition to angiostatin, other antiangiogenesis genes have been packaged into AAV shells for cancer therapy. Davidoff et al delivered a soluble, truncated form of the vascular endothelial growth factor receptor-2 (Flk-1) into liver via AAV vectors. Of the 15 mice, 10 receiving the vectors did not develop renal tumors, while the five remaining mice exhibited a tumor growth delay.[128] Shi et al demonstrated that intramuscular injection of rAAV2/human endostatin vectors led to sustained serum transgene expression which significantly inhibited tumor vessel formation and tumor growth.[130] Tumor growth was also significantly reduced following transduction with AAV2/Timps vectors (tissue inhibitors of matrix metalloproteinases) into Kaposi'sarcoma engrafted nude mice.[133]

Since various antiangiogenesis proteins prevent tumor vascularization through different mechanisms, it has been suggested that a combination of therapies utilizing different antiangiogenesis agents potentiates an additive inhibitory effect on tumor growth.[134–136] Recently, Ponnazhagan used this approach and delivered both endostatin and angiostatin in a single AAV2 shell. A synergistic protective efficacy was demonstrated on tumor development.[137] Consistent with the above observation, we injected AAV1/murine angiostatin and AAV1/murine endostatin into xenografted mice intramuscularly. The combination of endostatin and angiostatin gene therapy suppressed LLC cells growth more than either endostatin or angiostatin alone in the xenograft tumor mice, with tumor growth inhibition of 91, 62, and 82%, respectively.[138]

Immunotherapy

Tumor immune therapy has been studied for over a century. Gene therapy for cancer has brought new optimism to this field by targeting both immune effector cells and tumor cells for gene transfer. Many approaches and strategies have been exploited to use AAV vectors to deliver genes to enhance the immune response against tumors through targeting either tumor cells or nontumor cells.

Interferon (IFN) has an antitumor effect by directly inhibiting tumor cell proliferation or immune modulation. In an early study, recombinant AAV2 viruses encoding a synthetic type I IFN gene (IFN-con1) were used to infect various human tumor cell lines. Tumor growth was not observed up to 3 months after these transduced cells were inoculated into nude mice; whereas mice receiving nontransduced cells developed tumors within 7–10 days. When a mixture of transduced and nontransduced cells was injected into mice, tumors developed slowly and then completely regressed. Tumor regression was also demonstrated when mice with an established Eskol tumor were treated with AAV/IFN-con1-transduced 293 cells. These results implicate that the human IFN-con1 gene delivered by AAV vectors has antitumor effects both directly and by tumor-targeted gene therapy.[139] Intratumoral injection of AAV/IFN-β vectors completely inhibited the growth of engrafted gliomas.[140] Recently, Mohr et al used AAV2 vectors to deliver tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo2L), a member of the TNF superfamily of cytokines involved in various immune responses and apoptotic processes, into human colorectal cancer cells. The tumor growth in mice was significantly inhibited by delivery of AAV2/TRAIL vectors.[141]

Vaccination represents a very promising alternative immunotherapy for cancer. Transduction of B cells from chronic lymphocytic leukemia (CLL) patients with AAV2/CD40 ligand led to the upregulation of the costimulatory molecule CD80 on both infected and noninfected CLL cells, and induced specific proliferation of HLA-matched allogeneic T cells, indicating the possibility of vaccination in cancer patients using tumor cells infected with AAV.[142] Liu et al explored a potential vaccine to use vectors encoding a dominant HPV16-E7 cytotoxic T-lymphocyte (CTL) epitope and a heat–shock protein in human papillomavirus (HPV)-induced tumors. A potent antitumor response against challenge with an E-7-expressing syngeneic cell line in immunocompetent mice was demonstrated after intramuscular injection of AAV vectors

encoding the fusion protein. Both CD4- and CD8-dependent CTL activity was induced *in vitro*.[143] Dendritic cells (DCs), the most potent antigen-presenting cells, have also been used as targets for cancer gene therapy. Even though AAV vectors do not appear to infect DCs following intramuscular injection, several recent studies have indicated that immature DCs were transduced by AAV.[144–147] DCs infected with an AAV vector containing the HPV-16 E6 or E7 gene induced a strong CTL response against primary cervical cancer cell lines after 7 days of priming. Tumor cell killing was significantly blocked by the addition of anti-MHC class I antibodies, indicating CTL function via the MHC class I-restricted killing. In addition, high levels of CD8 + T cells were induced by transduced DC with high levels of CD80.[148,149] Based these observations, AAV-transduced tumor cells or DCs induce very strong CTL response to tumor cells.

Suicide gene therapy and enhancing chemotherapy

The basic principle behind suicide gene therapy is the selective intratumoral activation of a nontoxic drug by specific transfer of the activating transgene into tumor cells. Using an albumin promoter and an α fetoprotein enhancer, AAV-mediated delivery of the herpes simplex virus thymidine kinase (*HSV-TK*) gene can selectively kill α-fetoprotein-positive hepatocellular carcinoma cells in a mouse model,[150] and a bystander effect was demonstrated followed by the administration of ganciclovir (GCV).[151]

Similar *in vivo* therapeutic effects of AAV-mediated delivery of the *HSV-TK* gene have also been reported in an experimental glioma model and a human oral squamous cell carcinoma.[152,153] It is well known that irradiation can enhance the transgene expression of AAV vectors via increased second-strand synthesis of the AAV genome. Kanazawa et al used a combination of irradiation and the *HSV-TK* gene delivered by an AAV2 vector to efficiently kill a human maxillary sinus cancer cell line *in vitro*.[154] In addition, the same group demonstrated that topoisomerase inhibitors can also enhance the cytocidal effect of AAV/*HSV-TK* on cancer cells by increasing second-strand synthesis of AAV vectors.[155]

Autologous peripheral blood progenitor cell (PBPC) transplantation has been used for treatment of many solid tumors in patients; however, PBPC contaminated with tumor cells may give rise to relapse following myelo-ablative therapy and PBPC transplantation. *In vitro* experiments have revealed that AAV2 transduces some tumor cells very efficiently but does not transduce human hematopoietic stem/progenitor cells.[156,157] Based on this finding, AAV2 vector-mediated gene transfer may be used to purge contaminating tumor cells from the hematopoietic stem cell population prior to transplantation. Fruehauf et al found that AAV2 vectors efficiently infect human HS-1 and HT 1080 sarcoma cells (more than 95%), while primary human mobilized peripheral blood progenitor cells are more resilient to transduction by AAV2. Transplantation of these sarcoma cells transduced with AAV2/TK vectors into immunodeficient mice resulted in a greater than 5 month survival (AAV2/TK-ganciclovir group), compared to only 3 weeks of survival in the control group.[158]

The development of multiple drug resistance (MDR) is a major obstacle to chemotherapy. MDR is associated with overexpression of the P-glycoprotein; P-glycoprotein is a 170-kDa transmembrane ATPase that exports chemotherapeutic drugs from cells. Many approaches have been exploited to reverse the MDR phenotype. We have used scAAV2 vectors to successfully deliver hairpin siRNA into multidrug-resistant human breast cancer and oral cancer cells, and dramatically reduce P-glycoprotein expression levels resulting in substantial reversal of the MDR phenotype in the cells.[159]

Repair of tumor cells

The development of malignant tumors is caused by imbalanced regulation of two groups of genes: oncogenes and tumor suppressor genes. Tumor suppressor genes are involved in cellular checkpoint control, preventing the passage of cells with damaged DNA or other cellular damage through the cell cycle. Transfer of wild-type (wt) p53 cDNA into cancer cells can suppress the tumor phenotype *in vitro* and *in vivo*. rAAV/p53-mediated transduction inhibited the growth of neoplastic cells with G1–S arrest and also mediated cytotoxicity with apoptosis. Tumor growth in three of five animals was completely inhibited after direct injection of rAAVp53 into H-358 tumors implanted subcutaneously in immunodeficient nu/nu mice,[160] which suggests the efficacy of rAAV-mediated phenotypic correction at the molecular level. The function of telomerase is to maintain and stabilize the integrity of telomeres. Activated telomerase is detected in many tumor cells. Zhang et al delivered telomerase antisense RNA into MCF-7 cells using a hybrid adenovirus/AAV vector. The telomerase activity was significantly suppressed, leading to reduced colony formation and cell proliferation as well as induction of tumor cell apoptosis.[161] Dumon et al used AAV to deliver the tumor suppressor gene Fragile histidine triad (FHIT) to human pancreatic cells and demonstrated slow tumor growth and long-term survival in a mouse model.[162]

Suppression of E6/E7 oncogene expression can reverse the transformed phenotype in cervical carcinomas. The monocyte chemoattractant protein-1 (MCP-1) indirectly suppresses E6/E7 gene expression and is absent in HPV-positive cervical carcinoma cell lines. AAV-2 vectors carrying the MCP-1 gene were used to transduce HPV16-or HPV18-positive cervical carcinoma cell lines (HeLa or SiHa, respectively). The expression of human MCP-1 strongly inhibited the development of tumors derived from either HeLa or SiHa cells transduced *in vitro* with AAV2 vectors. Similar results were also achieved after *in vivo* delivery of AAV2/MCP-1 into SiHa-derived tumors.[163]

Combined gene therapy with AAV

Combination gene therapy can improve antitumor capacity. Early work has shown that transduction of U-251SP human glioma cells *in vitro* with a bicistronic AAV vector containing both the TK and human interleukin-2 genes (AAV-tk-IRES-IL2) rendered these cells susceptible to GCV treatment and allowed the cells to produce IL-2 in a dose-dependent manner. After stereotactic delivery of AAV-tk-IRES-IL2 transduced glioma cells into nude mice, the tumor volume following GCV administration was reduced by 35-fold compared to controls.[164] A similar strategy used by another group examined the effects of the AAV2/TK/IL-2 vector in the mouse hepatocellular carcinoma cell line Hepa 1–6. Hepa 1–6 cells, transduced with either AAV2/TK/IL-2 or AAV2/TK, were injected into both nude mice and immunocompetent C57L/J mice. Tumor cells that had been transduced with AAV2/TK/IL2 were more susceptible to GCV treatment than tumor cells transduced with only TK. In the absence of GCV treatment, all mice inoculated with AAV/TK/IL-2-transduced cells were tumor free by day 24 postinoculation, indicating that IL-2 alone has a profound effect on tumor killing. The tumor-killing effect of AAV-mediated TK/IL-2 gene transfer was further studied by mixing TK/IL-2- or TK-transduced tumor cells with unmodified tumor cells and then evaluating tumor growth with and without GCV treatment. The optimal result was observed from the TK/IL-2-transduced group without GCV treatment. Animals receiving 10% transduced cells with no GCV treatment saw total clearance of the tumor as well as long-term protection against rechallenge with tumor cells in 50% of the group. However, when the group with TK/IL-2-transduced tumor cells was treated with GCV, the antitumor effect of TK/IL-2 was decreased. This suggests that GCV treatment results in the short-term IL-2 expression by the early killing of AAV-tranduced tumor cells. It was concluded that TK/IL-2 induces a stronger tumor-killing effect than *HSV-TK* with administration of GCV, but that the tumor killing of TK/IL-2 is more effective without addition of GCV.[165] Janouskova et al attempted

to investigate the antitumor effect with the combined delivery of a suicide gene and immunostimulatory gene to cancer cells. TC-1 cells, HPV-16-transformed C57BL/6 mouse cells, were infected with AAV2/TK and either AAV2/MCP-1 or AAV2/B7.1 *in vitro*. These cells were then transplanted into mice and GCV was administered. None of the mice treated with the combination of AAV2/TK and AAV2/B7.1 or AAV2/MCP-1 developed tumors. The tumor-free mice were rechallenged with untreated TC-1 cells 54 days after tumor engraftment, and it was found that the tumor resistance rate was related to immunostimulatory gene delivery and the utilization of GCV. In agreement with the results of Su et al, the best protection was observed in mice preinoculated with TC-1 cells transduced with either B7.1- or MCP-1-expressing rAAV without GCV administration.[166]

An attempt to combine antiangiogenic therapy and immunotherapy against tumor growth has recently been carried out by Sun et al. Mice implanted with B7.1-engineered EL-4 tumor cells by AAV transduction demonstrated slow tumor growth and resisted rechallenge with unmodified tumor cells. However, these mice were unable to resist the challenge with over burden of tumor cells, which was overcome by intraportal injection of AAV/angiostatin before rechallenge. The study implicates that the synergistic effect against tumor growth can be achieved by combination of immunotherapy and anti-angiogenesis therapy with AAV vectors.[167]

Targeted cancer gene therapy with AAV

For the safety and efficiency of cancer gene therapy with rAAV, the development of targeted-AAV for cell-specific delivery is critical for targeting tumor cells directly *in vivo* to enhance local delivery and effective suppression of tumor growth. Two approaches have been exploited to express transgenes in specific tumor cells delivered by AAV vectors: tumor cell surface targeting (transductional) and transcriptional targeting with cell-specific enhancers/promoters.

With the discovery of the AAV crystal structure and the identification of the heparin binding motif on AAV capsid,[14,15,168] it becomes quite feasible to retarget AAV transduction by modifying the capsid for cancer gene therapy. Grifman et al incorporated the tumor-targeting sequence NGRAHA into the AAV capsid. This targeting sequence contains the peptide motif, NGR, responsible for binding CD13, a receptor expressed in angiogenic vasculature and in many tumor cell lines. The vectors containing the NGRAHA sequence were able to transduce Kaposi sarcoma (KS1767) cells and the embryonal rhabdomyosarcoma cell line (RD) 10–20 fold better than wt AAV2.[91] Incorporation of an Arg-Gly-Asp (RGD)-containing peptide in the AAV capsid enabled this mutant AAV to transduce integrin-expressing cells independently of heparin binding and led to increased transduction in tumor cell lines which express integrin, but few heparin-binding receptors.[169]

Others have attempted to use specific tissue enhancer/promoters to achieve tumor-specific AAV-mediated gene expression. In one approach, the promoter of the glucose transporter isoform 1 (GLUT1) gene was utilized to drive the enhanced green fluorescence protein (EGFP) and the HSVtk gene expression. EGFP expression under the control of the GLUT1 promoter element (rAAV/GTI-1.3egfp) was restricted to tumor cells and oncogene-transformed cells following infection with AAV2/GLUT1-EGFP-TK. Tumor remission was achieved after TK-expressing tumors were treated with GCV.[170] Based on difference on hypoxia between tumor cells and normal cell, Ruan et al used AAV vectors to deliver transgene Epo to human brain tumor cell lines U-251MG and U-87MG under the control of hypoxia-response elements (HRE), which are activated by the transcriptional complex hypoxia-inducible factor-1. A 79–110-fold increase of Epo expression was observed with anoxic condition after transduction with AAV2 regulator vectors.[171]

Recently, Nicklin et al combined AAV vector surface modification and transcriptional regulation for cancer gene therapy. The SIGYPLP-targeting peptide was inserted into AAV virus capsids, conferring the ability to transduce six of 12 tumor cell lines (C8161, PC-3, G-CCM, MKN-45, LnCAP and A549) regardless of native viral tropism. Furthermore, the cancer-specific promoter FLT-1 was found to be active in three of these six cell lines (PC-3, A549 and MKN-45). These results suggest the potential application for dual targeting at transduction level and transcriptional regulation level in cancer cells.[172]

Conclusion and future prospects

AAV vectors can efficiently initiate sustained transgene expression *in vivo* and appear to be safe. With the identification of different serotypes and recent progress in the improvement of AAV vectors, such as dual vectors to overcome the limited packaging capacity, self-complementary vectors to increase the level and onset of transgene expression, and capsid modifications to mediate cell specific transduction, it will be possible in the future to design more specific and efficient therapies for use in the cancer treatment arena.

Most of experiments *in vitro* and *in vivo* with AAV vectors were performed in tumor cell lines, so it is imperative that more work should be carried out in the primary tumor cells in the future since the transduction efficiency with AAV vectors may be different between cell lines and primary cells. Although researchers have found that some tumor cells are not permissive to AAV infection, with the discovery of AAV crystal structure and development of receptor retargeting AAV vector, direct targeted tumor cells with engineering AAV vectors will provide promising approach with specificity and efficiency for cancer gene therapy. It is clear that there is wide variation in transduction efficiencies among different cell types using different AAV serotypes. The identification of a possible cellular receptor and coreceptors for AAV serotypes will expedite the application of AAV as a gene therapy vector. It is becoming evident that additional developments to achieve high infectivity will be predicated on effective utilization of AAV-based vectors in cancer gene therapy using such techniques as maker rescue and mixed capsid vectors.

For receptor retargeting, it is very important to find an optimal ligand or targeting receptor, since the length and sequence of the insertion may result in profound alterations of the three-dimensional capsid structure.[89] Using AAV vector-phage display may help to find an optimal inserted peptide.[173,174]

In addition to AAV targeting studies, the understanding of tumor development at biological and molecular biological levels will lead to the discovery of strong, efficient, and specific enhancers/promoters in tumor cells. Utilization of regulatory systems will avoid the undesired side effect of systemic transgene expression delivered by AAV vectors for immune-modulation and antiangiogenesis.[175,176]

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