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Review

Adeno-associated Virus for Cancer Gene Therapy[1]

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Introduction

Gene therapy for cancer offers novel treatment paradigms that will eventually lead to the destruction of tumor cells in patients with solid and hematopoietic malignancies. Major cancer gene therapy approaches that directly target tumor cells include chemosensitization, cytokine gene transfer, inactivation of proto-oncogene expression, replacement of defective tumor suppressor genes, and transduction of oncolytic viruses. A vast majority of these approaches have been attempted using adenoviral vectors and to a lesser extent, retroviral vectors. AAV[3]-based vectors are recently emerging nonpathogenic vectors with potential for cancer gene therapy. AAV belongs to the group of human Parvovirus with a single-stranded DNA genome. The identification of AAV as a viral entity was reported 3 decades ago (1). For a replicative life cycle, AAV requires the presence of helper viruses and, hence, is also known as dependovirus. The helper functions are normally provided by adenovirus, herpesvirus, or vaccinia virus (2–4). In the absence of a helper virus, AAV integrates into host genome and establishes a latent cycle. When a latently infected cell encounters superinfection by any of the helper viruses, the integrated AAV genome rescues itself and undergoes a productive lytic cycle. Although a plethora of studies on the biology of AAV has been published in the past 3 decades, a realization of the potential of AAV as a gene-transfer vector began about 15 years ago (5, 6). Since then, a number of studies have shown significant progress in both the application of AAV-based vectors in gene therapy for a variety of diseases and the technology of high-titer, contamination-free rAAV production. Over the last few years, several *in vivo* studies using rAAV have shown efficacious results in the treatment of multiple diseases in animal models and in human clinical trials (7–17). Furthermore, rAAV does not encode any wt viral genes and, hence, is less immunogenic compared with other commonly used viral vectors (18, 19). Interestingly, wtAAV has also been identified as possessing antioncogenic properties (20–21). Although rAAV vectors are relatively less studied in cancer gene therapy, those reported thus far indicate their potential in cancer gene therapy targeting the tumor cells. In addition, although most of the above-mentioned strategies target tumor cells directly for increasing therapeutic benefit, targeting normal cells that regulate key events conducive to tumor growth is becoming a promising alternative in cancer therapy. For direct targeting of tumor cells, although a vector need not possess characteristics of long-term expression or the ability to integrate into the host genome, these features may be beneficial in strategies aimed at targeting normal cells, such as tumor endothelium, that exert a sustained control over tumor growth. In this regard, AAV remains a promising vector for cancer gene therapy. We describe here the biology and potential of rAAV as applied to direct and indirect cancer gene therapy approaches.

Molecular Organization of AAV

AAV contains a genome of 4679 bases of single-stranded DNA (22). Both positive and negative strands of the viral genome are equally packaged in AAV capsids (23). The genome of AAV encodes two proteins, namely Rep, which is a nonstructural protein involved in rescue and replication of the virus, and Cap, which forms icosahederal capsid within which the replicated genome is packaged. There are three different promoters that have been identified in the AAV genome. On the basis of their relative position in map units, they are named as p5, p19, and p40 (24–27). Whereas transcripts from the p5 and p19 promoters produce four different species of Rep proteins by alternate splicing, transcript from p40 produces three different capsid proteins (Fig. 1). Rep68 and Rep78 are produced from p5 promoter as spliced and unspliced forms, respectively, and Rep40 and Rep52 are produced from the p19 promoter similarly (28, 29). Whereas Rep68 and Rep78 are known to play vital roles in replication of the AAV genome (30–32), regulating transcription of AAV promoters (33–35), and directing site-specific integration of the AAV genome into chromosome 19 in human cells (36), Rep52 and Rep40 are important for the production of single-stranded vector genome (37–39). It has been reported that site-specific integration of rAAV is achievable by complementing Rep in the rAAV genome (40, 41).

The *Cap* gene encodes three different capsid proteins namely VP-1, VP-2, and VP-3. Although all three capsid proteins are transcribed from the p40 promoter, different initiation codons are used in their translation. Whereas VP-1 and VP-3 use ATG as the start codon, VP-2 uses ACG as the initiation codon (22). Among the three capsid proteins, VP-3 is the predominant capsid protein and represents ~90% of the icosahederal structure. Although complete assembly of viral capsids is achievable with only VP2 and VP3, mutations in NH_2-terminal region unique to VP-1 produced DNA-containing vector particles with significantly reduced infectivity (23, 42), which indicated a need for all three capsid proteins for optimal transduction. Different serotypes of AAV have been identified and shown to contain variations in the amino acid sequence of capsid protein, which suggests their potential utility in gene therapy applications (43, 44).

In addition to the *Rep* and *Cap* genes, the AAV genome also contains two ITRs on either end of the genome that are ~145 bases in length each. The ITRs are sole elements required for rescue, replication, packaging, and integration of AAV (22, 45). The ITRs are rich

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[3] The abbreviations used are: AAV, adeno-associated virus; rAAV, recombinant AAV; wt, wild type; ITR, inverted terminal repeat sequence; IL, interleukin; HPV, human papillomavirus; TK, thymidine kinase; HSV, herpes simplex virus; GCV, ganciclovir; DC, dendritic cell; MCSF, macrophage colony stimulating factor; GM-CSF, granulocyte/macrophage-colony stimulating factor.

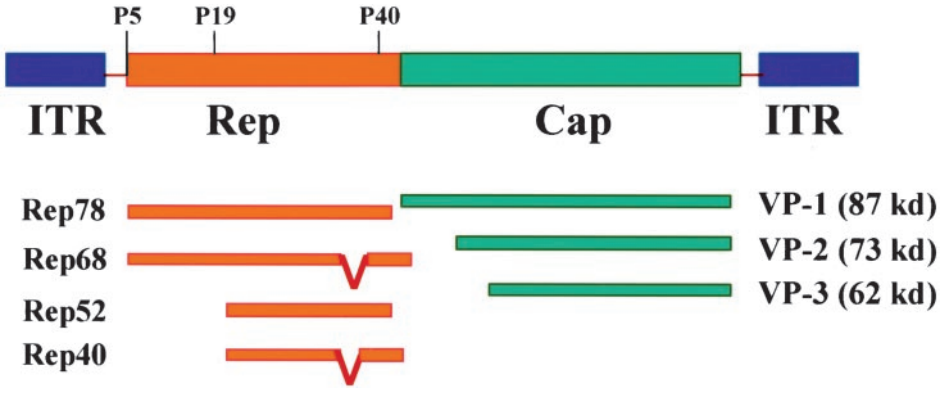

Fig. 1. Genomic organization of AAV. The transcripts from promoter P5 give rise to Rep78 and Rep68 by alternate splicing whereas those from P19 give rise to Rep52 and Rep40 by similar mechanism. The three capsid proteins, VP-1, VP-2, and VP-3 are synthesized using transcripts from the promoter P40. *kd*, M_r of each capsid protein in thousands. *ITR*, the ITRs necessary for viral replication, rescue, packaging, and integration.

in GC bases and form a hairpin structure with three complementary domains that form a double-stranded structure (22, 45). It is this folding-over that forms a primer for leading-strand synthesis, which is essential for transcription and integration on viral infection to host cell nuclei. The ITRs also contain a six-base sequence known as terminal resolution site, which is recognized by Rep68 and Rep78 to create a single-stranded nick preceding viral DNA replication. In addition to these characteristics, recent reports have also indicated potential promoter activity of AAV-ITRs (46).

Production of rAAV

Initial cloning of the AAV genome into a plasmid vector facilitated a wide range of molecular manipulations that led to the understanding of several key events in AAV biology (47). The crucial role of ITRs in the AAV life cycle had been shown initially in experiments using rAAV plasmids containing heterologous gene sequences flanked by AAV-ITRs. Transfection of the rAAV plasmids into human cells resulted in successful rescue, replication and packaging of infectious mature virions by transcomplementing AAV *Rep* and *Cap* genes from a nonrescuable plasmid and by coinfecting with adenovirus to provide helper functions (18). After this observation, a variety of studies have shown successful packaging of the rAAV genome. Initial methods of rAAV production involved cotransfection of a AAV helper plasmid (pAAV/Ad; Ref. 18) along with rAAV plasmid containing heterologous genes flanked by AAV-ITRs into 293, HeLa, or KB cells and subsequent infection of these cells with wt adenovirus. Approximately 48–72 h after the transfection/infection, the cells were lysed, and extracts containing rAAV were used after heat inactivation at 56°C to destroy contaminating adenovirus. DNaseI digestion was used to remove unencapsidated and input plasmid genome.

Additional modifications in rAAV production and purification steps that involved (*a*) generation of packaging cell lines (48–51), (*b*) cloning of helper plasmids containing necessary adenoviral genes to eliminate any wt adenovirus in AAV preparations (52–55), (*c*) gradient ultracentrifugation methods that allowed precise isolation of rAAV based on buoyant density (56), and (*d*) purification using affinity columns and high-performance liquid chromatography have resulted in high-titer rAAV yields necessary for *in vivo* studies (56). These advancements have resulted in obtaining 10^{12}-10^{13} particles of rAAV routinely from $\sim 10^9$ cells. The physical and infectious titers of rAAV preparations are determined by quantitative slot blot analysis (57), infectious center assay (56), and quantitative PCR (58). An outline of rAAV production is depicted in Fig. 2. Despite these advances, further refinement in the production and purification steps are warranted for optimal utilization of rAAV in human clinical trials. Although advancements in current AAV packaging methods have eliminated any possibility of wt adenovirus contamination in rAAV preparations, recombination of homologous regions present in rAAV and AAV helper plasmids still results in a very minimal amount of wtAAV in rAAV preparations even by the most advanced and current methods of packaging.

Unlike other gene therapy vectors currently used, AAV in its wt form is a nonpathogenic virus as well as replication-incompetent. Despite these innate "safety" features, it will be necessary in the future

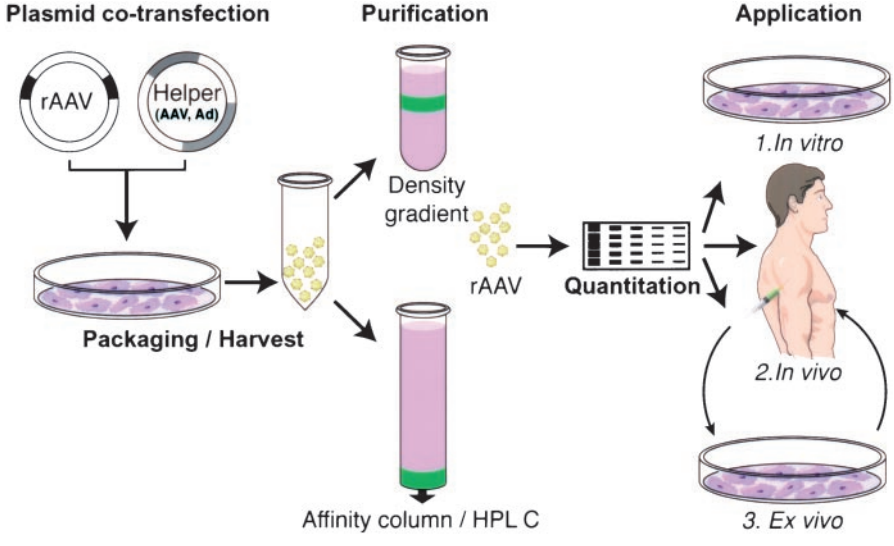

Fig. 2. Strategy for the production of rAAV. A recombinant plasmid containing gene(s) of interest, subcloned within the ITRs of AAV is cotransfected with a recombinant helper plasmid containing the wtAAV and adenoviral genes necessary for the rescue, replication, and packaging of rAAV in 293 cells. Approximately 48–60 h later, the cells are lysed, and crude extract containing the rAAV and cellular proteins is cleared and subjected to further purification, either through density gradient centrifugation or through column chromatography. The physical and infectious particle titers of the purified rAAV are determined, and the purified virus is subsequently used for *in vitro* and *in vivo* studies.

to devise strategies such as development of high-efficiency packaging cell lines to totally eliminate any wtAAV contamination in recombinant vector preparations. Another important consideration in improving rAAV production is reducing the number of noninfectious defective particles in packaging and purification steps. Although it may be difficult to physically separate infectious and defective particles entirely by any of the purification methods currently in vogue, because several defective particles also exhibit similar capsid characteristics, it may still be possible to alter molecular events of packaging, which could promote complete replication of the rescued molecules and further identification of packaging signal(s). Furthermore, elimination of strenuous steps in purification such as extensive ultracentrifugation for several hours may also help to improve overall quality of the vector. It is also interesting to note that the titers of wtAAV by the same methods of production yield at least a 1–2 log increase. The only difference in packaging of wtAAV by the plasmid transfection method is that the Rep and Cap proteins of AAV are provided in *cis* from the rescuing/replicating genome, whereas in the packaging of rAAV, these proteins are supplied in *trans*. Thus, future studies to understand the molecular coordination between replication and packaging may result in still higher vector yields. These determinants may have a direct implication for future gene therapy applications of rAAV.

The Potential of rAAV as a Vector for Gene Therapy

Several recent studies have indicated the efficacy of rAAV as an alternative to more commonly used adenovirus and retrovirus-based vectors for human gene therapy. As with other vectors, there are both advantages and disadvantages to the potential application of rAAV in gene therapy. Whereas some of the major advantages of AAV vectors include stable integration, low immunogenicity, long-term expression, and ability to infect both dividing and nondividing cells, the major limitations include variations in infectivity of AAV among different cell types and the size of the recombinant genome that can be packaged. Furthermore, initial *in vitro* studies indicated the ability of rAAV to infect a variety of human and animal cell types of different origin. Subsequently, the *in vivo* efficacy of rAAV was proven in murine and nonhuman primate models using a variety of candidate genes and target tissues [for a detailed list of candidate genes and different target tissues, please refer to the review by Snyder (59)].

Although studies have shown the efficacy of AAV-mediated gene therapy in different human and mammalian cell types, the most efficient vector transduction has been reported in skeletal muscle and brain followed by hepatocytes *in vivo*. Long-term undiminished expression of the AAV transduced genes has been reported for over 1½ years after i.m. delivery (60). Studies in the last few years have identified several possible reasons for variations in transduction efficiency among cell types.

The identification of heparan sulfate proteoglycan as the cellular receptor (61) and possibly fibroblast growth factor-1 [FGFR1 (62)] and $\alpha V\beta 5$ integrin (63) as coreceptors account for the primary event of viral entry. Subsequent to infection, AAV is transported to the nucleus within a short time (64–66) and uncoating of the capsid releases the vector genome in the host cell nucleus. Because AAV is a single-stranded DNA-containing virus, efficient conversion of the single-stranded structure to double-stranded forms is a prerequisite for additional events such as transcription and integration. A few events are known to facilitate this process. Because both positive and negative strands are packaged equally in AAV preparations, it is likely that transcription observed at an early stage after vector transduction could result from the annealing of positive and negative strands. An additional event that results in the double-stranded structure of the transgene is second-strand synthesis of the vector genome from an original single-stranded template (67, 68). Several stimuli such as UV, heat shock, hydroxyurea, γ-irradiation, and Ad E4-ORF6 are known to promote conversion of single-strands into double-strands (69–71).

Initial *in vivo* studies have reported that steady-state expression of the transgene occurs only after a few weeks of vector delivery. This was attributed to delay in the conversion of the single-stranded genome into a double-stranded structure (72–76). However, recent studies indicate that this conversion occurs within a few days of transduction (77). Further, it has been well established that the AAV genome integrates into the host chromosome as concatemers rather than as monomers. Reports also indicate that the rate of formation of concatemers correlates with expression levels *in vivo* (73). In addition to the required conversion of the single-stranded genome to a transcriptionally active substrate, certain cellular events have also been reported to play a role in the level of transgene expression including topoisomerase inhibitors such as etoposide and tyrphostin, which have been shown to increase expression of AAV transgene (78). Recently, the possible role of a host cell phosphoprotein termed ss-DBP has been reported to exert an effect in AAV-transgene expression (79). Furthermore, a role for epidermal growth factor receptor tyrosine kinase has been implicated in this process (78). It has also been reported that expression of rAAV transgene is higher in cells that are actively dividing. However, this property has been documented only in certain cell types, such as primary human fibroblasts and hematopoietic cells (70, 80). On the other hand, high-level expression of rAAV transgenes has been reported in skeletal muscle and brain, which are generally nondividing (10, 60, 72, 75, 81–83).

Taken together, it remains possible that in different cell types, the mechanisms that regulate expression of AAV-encoded genes are different. Whereas in actively dividing cells an enhanced metabolic rate may promote events that regulate DNA replication and gene expression, in nondividing cells, recombination and/or ligation of the vector genome to form concatemers may result in active expression (84). Because, in nondividing cells, there is less likelihood of dilution of the transduced AAV-DNA and possible infection of higher multiplicity of vector, these events are more likely to result in enhanced gene expression. It has been postulated that in nondividing cells, episomal concatemers may also contribute to higher gene expression apart from integrated copies of the vector. Recent reports indicate that single-stranded DNA disappears from the liver within 5 weeks of vector administration, which suggests not only an efficient conversion of the single-stranded DNA but also the presence of high molecular concatemers (85). Furthermore, that the expression levels correlated with the amount of concatemers present in these studies suggested the importance of concatemer formation in transgene expression *in vivo*.

In addition to the conformational changes required for transgene expression and integration, the nature of the regulatory elements including promoters appears vital to the levels of transgene expression. Most of the AAV-mediated *in vivo* studies reported to date have been carried out using human cytomegalovirus immediate early promoter. However, endogenous promoters, such as α-globin promoter (86), β-actin promoter (87), and IL-2 promoter (88), have been used in certain gene therapy contexts with AAV. Use of such tissue-specific promoters represents one strategy to achieve restricted expression of the transgene in target cells. Furthermore, because native promoters and other regulatory elements are not foreign to the target cells or the immune system, promoter inactivation may be reduced over time, allowing longer expression of the transgene. Chimeric promoters such as chicken β actin/creatine kinase and human skeletal actin/cytomegalovirus have also been shown to be efficacious in inducing high-level expression of the transgene compared with individual promoters (89, 90). Regulated-expression of transgene has also

been reported in AAV-mediated transduction *in vivo*. Regulated expression of growth hormone and erythropoietin genes have been successfully achieved with AAV vectors by using rapamycin and tetracycline systems respectively (91–93). Thus, many variables determine the efficacy of rAAV-mediated gene transfer and expression.

Gene Therapy as an Alternate Approach for Cancer Treatment

Gene therapy offers a potentially useful approach for the treatment of cancers because a variety of genes controlling molecular processes can be introduced by gene transfer, which can in principle arrest tumor growth, angiogenesis, invasion, and/or metastasis. However, several major obstacles need to be overcome for these approaches to be successful. First, our understanding of the molecular processes that lead to tumorigenesis and neoplastic progression is far from complete. Thus, therapies directed at the molecular events known to promote tumor progression may be limited by an incomplete understanding of the underlying mechanisms. Second, direct gene delivery to cancer cells *in vivo* is highly limited by chaotic blood supply. Despite these limitations, gene therapy has shown promising results in several preclinical studies with a variety of vectors and also to some extent in human clinical trials (94).

Currently available cancer gene therapy methods can be broadly divided into those that exert immediate cytotoxicity on tumor cells and those that initiate regulatory events that lead either to correction of underlying defects in tumor cells at a molecular level or to enhancement of the ability of the host immune system to innately recognize tumor cells for T-cell-mediated killing. A majority of cytotoxic gene therapy involves the delivery of genes that encode enzymes such as TK and cytosine deaminase, followed by the administration of nontoxic prodrugs, which are eventually converted to cytotoxic intermediates in the cells that express the transgene.

Genetic correction of a molecular defect in tumor cells has also been attempted. The identification of genes that contribute to oncogenic transformation of cells presents an opportunity to use these genes and their products as treatment and potential prevention targets. The genes that are implicated in carcinogenesis include dominant oncogenes such as members of the *ras* family and tumor suppressor genes including *p53* (95, 96). Although inactivation of dominant oncogene products at the transcriptional level with antisense RNA may block their production, proper expression of tumor suppressor genes through gene transfer appears to be required to suppress the growth of tumor cells or to lead to apoptosis and necrosis.

Different gene therapy approaches are being used to enhance the host immunity against tumor cells. One strategy has been to vaccinate "the host" with tumor cells that have been modified *ex vivo* by the transfer of genes that encode cytokines, tumor-associated antigens or portions of the MHC. A variety of such molecules have shown promising results in controlling tumor growth in animal models. These include IFN-α, IFN-γ, tumor necrosis factor-α, MCSF, GM-CSF, IL-1, IL-3, IL-5, IL-6, IL-7, IL-10, and IL-12 (97–100). The *in vitro* growth characteristics of many tumor cells are not affected by cytokine gene transfer, thus confirming the supposition that the suppression of tumorigenicity *in vivo* is caused by interaction of the host immune defense system in addition to the expression of these molecules (101). Another approach has been to immunize against a cloned tumor-specific antigen. Although initial approaches of genetic immunization targeted muscle cells for expression, processing, and presentation of the antigen, subsequent studies have effectively used antigen-presenting cells, particularly the DCs, for transduction with tumor-associated antigen genes (101–107). Furthermore, transfer of costimulatory molecules such as B7.1 and B7.2 have also shown significant enhancements of host immunity (108). There has also been considerable interest in the use of gene transfer to enhance tumor homing or tumor cell-killing by adoptive transfer of *ex vivo* expanded tumor-infiltrating lymphocytes (109).

Antioncogenic Properties of AAV

The antitumor effects of AAV had been initially reported within a few years of identification of the virus. One example of this was the realization that infection of HSV-transformed hamster tumor cells with AAV delayed the appearance of palpable tumors and increased the survival time of the animals (110). Since then, several reports have confirmed the inhibition by AAV of viral oncogenesis by a variety of DNA viruses, including bovine papillomavirus-1 (111), HPV-16 (35, 112, 113), and EBV (114). Evidence from several reports also suggested that AAV infection might protect against human cervical cancer, in part by interfering with HPV-induced tumorigenesis (115), although studies of Strickler *et al.* (116) reported a lack of correlation between AAV infection and cervical tumorigenesis in a Jamaican population.

Elucidation of the molecular mechanisms directing the antitumor properties of AAV identified a role for Rep78 in the inhibition of oncogenic transformation, specifically the down-regulation of human c-*fos* and c-*myc* proto-oncogene promoters by Rep78 (117). Inhibition of HPV-16 P97 promoter activity (115) may partially account for the tumor inhibitory property of Rep78 in cervical cancer cells. A recent study (118) reported that whereas Rep78 and Rep68 inhibited the growth of primary, immortalized, and transformed cells, Rep52 and Rep40 did not. Furthermore, Rep68 induced cell cycle arrest in G_1 and G_2 with elevated cyclin-dependent kinase inhibitor p21 and reduced cyclin E-, A- and B1-associated kinase activities. Rep78 was also found to arrest the cell cycle, preventing S-phase progression by binding to the hypophosphorylated retinoblastoma protein (118). The regulatory differences between Rep78 and Rep68 has now been mapped to the COOH-terminal zinc finger domain of Rep78. These studies indicate that Rep proteins exert heterologous control at both the molecular and cellular levels in inhibiting tumor growth. Despite the significance of Rep78 and Rep68 in tumor-suppression, potential utilization of Rep as a therapeutic molecule is limited by its toxicity (119). Thus, additional advancements in highly tumor cell-specific delivery and/or expression of *Rep* gene is required before Rep can be used as a therapeutic molecule. Current advances in technology to identify both tissue-specific regulatory elements and candidate ligands/molecules for receptors that are overexpressed in tumor cells should lead to the development of transductional and transcriptional targeting of rAAV vectors encoding Rep as a therapeutic molecule in the future.

Molecular Chemotherapy Studies with rAAV

Delivery of a gene-encoded toxin into cancer cells to achieve tumor eradication is usually performed by indirect killing through activation by a prodrug. This approach has focused mainly on delivery of the *HSV-TK* gene. Expression of HSV-TK results in replicating tumor cells having enhanced sensitivity to nucleoside analogues, such as GCV or acyclovir. GCV is phosphorylated initially by TK and subsequently by cellular factors to a triphosphate form that becomes incorporated into cellular DNA (120). This inhibits both DNA synthesis and RNA polymerase activity, which results in cell death (120).

Although a majority of both preclinical and clinical gene therapy studies using molecular chemotherapy approaches have been conducted with recombinant adenoviral vectors, AAV-mediated *in vivo* studies have also indicated therapeutic benefits for tumor regression.

Selective killing of α fetoprotein-positive hepatocellular carcinoma cells by AAV-mediated gene transfer of *HSV-TK* gene was reported in a mouse model using an albumin promoter and an α fetoprotein enhancer (121). Further work by the same group also reported therapeutic efficacy and a bystander effect of AAV-mediated intratumoral delivery of the *HSV-TK* gene followed by treatment using GCV (122). Interestingly, in additional experiments, the same group also reported an enhancement of tumor-cell killing with a rAAV containing the *HSV-TK* gene along with *IL-2* gene compared with transduction of vector containing only the *HSV-TK* gene (123). Thus, it is possible to enhance antitumor effects by delivering two different therapeutic genes in the same vector. Although there is a size constraint in the packaging of foreign genes in rAAV, most of the therapeutic genes in the context of cancer therapy are well within the packaging limits of rAAV, either alone or in tandem. Similar *in vivo* therapeutic effects of AAV-mediated delivery of the *HSV-TK* gene has also been reported in an experimental glioma model (124).

Consideration of molecular chemotherapy strategies for selective killing of tumor cells suggests that integration of transgenes is not a desirable feature; hence, the choice of AAV-based vectors is not preferred as compared with nonintegrating adenoviral vectors. Furthermore, the efficacy of adenoviral infection in different tumor cells has been reported to be significantly higher than that with many other available gene therapy vectors. However, it has recently been reported that the efficiency of rAAV transduction of primary tumor material that is derived from malignant melanoma and ovarian carcinoma is significantly higher (>90%) than that seen in established tumor cells of the same derivation in culture (125). This observation suggests that it is possible to use rAAV in direct targeting of tumor cells for an effective killing by approaches such as molecular chemotherapy, cytokine gene transfer, and inactivation of proto-oncogene expression. In addition, studies by Su *et al.* (123), using an AAV-TK-IL-2 vector, reported the disappearance of the rAAV genome after GCV treatment and regression of the transduced hepatocellular carcinoma. Although rAAV integrates into the host genome, unlike transgene expression, integration of the vector does not occur immediately after transduction. Hence, GCV treatment after vector administration at an early time point should still achieve therapeutic benefit minimizing long-term retention of the transgene. Identification of tumor cell-specific ligands and use of tissue-specific promoters may also allow one to both transductionally and transcriptionally target rAAV intratumorally. Possible correction of malignant phenotype by rAAV-mediated *p53* gene transfer has also been reported recently (126), which suggests the efficacy of rAAV-mediated phenotypic correction at a molecular level.

AAV-mediated Long-Term Expression as a Potential Cancer Gene Therapy Strategy

It is now well established that tumor growth and metastasis are dependent on the recruitment of a functional blood supply by a process known as tumor angiogenesis, and indeed, the "angiogenic phenotype" correlates negatively with prognosis in many human solid tumors (127, 128). The establishment of angiogenic requirements for tumor growth led to the identification of several antiangiogenic molecules that potentially inhibit growth of tumor neovasculature (129). Antiangiogenic therapies devised thus far target different steps of the angiogenic process, ranging from the inhibition of expression of angiogenic molecules, through overexpression of antiangiogenic factors, to direct targeting of tumor endothelial cells using endogenous angiogenic inhibitors or artificially constructed targeting ligands (130).

Although a majority of preclinical and clinical antiangiogenic therapies to date have been conducted with purified antiangiogenic factors (131), gene therapy appears to be more powerful than other forms of antiangiogenic therapy. Potential advantages of antiangiogenic gene therapy are sustained expression of the antiangiogenic factors and highly-localized delivery (130). Despite these advantages, vector development still remains in its infancy for this form of therapy. Adenoviruses are again the most commonly used vectors for this strategy and, in several preclinical studies, have shown promise (132–136). Nonetheless, expression of antiangiogenic factors mediated by adenovirus-based vectors is limited by an effective host immune response and is also secondary to the episomal nature of the vector. AAV, on the other hand, possesses most of the salient features to be a desirable vector for antiangiogenic gene therapy.

The advantages of rAAV over other vectors for antiangiogenic gene therapy are multifold. First, AAV is a nonpathogenic vector with a limited host immune response. Second, AAV is an integrating vector; hence, long-term expression of antiangiogenic factors is possible *in vivo*. Third, most of the antiangiogenic genes are within the capacity to be cloned in AAV, either independently or in tandem. Provision of two different antiangiogenic genes from the same vector may yield added therapeutic benefits because different antiangiogenic factors may work through different metabolic pathways. Furthermore, undiminished long-term persistent expression of rAAV-encoded proteins has been reported in a variety of studies (8, 17). By a plasmid delivery, i.m. administered secretable endostatin, a biologically driven antiangiogenic factor, has been shown to provide therapeutic benefits in a murine model through systemic transport to a tumor site (137), which indicates a high likelihood of a similar strategy with rAAV. In addition, reports indicate that the efficacy of rAAV transduction to primary tumor cells is significantly higher when compared to efficacy in cell lines (125). Advances in the development of targeted-AAV for cell-specific delivery may well be used in future AAV-mediated antiangiogenic gene therapy applications that target tumor cells directly *in vivo* to enhance locoregional delivery and effective suppression of tumor growth. It has been reported recently by Nguyen *et al.* (138) that appropriate expression of human angiostatin and endostatin, leading to the inhibition of endothelial cell growth, was possible using rAAV vectors. Thus, further advances in AAV-mediated antiangiogenic gene therapy should see exciting results in future cancer gene therapy applications.

AAV Vectors for Immunotherapy

The potential of AAV vectors for cancer immunotherapy is evident from recent studies using cytokine gene transfer and *in vivo* immunization approaches (139–141). Active immunization with tumor cells transduced with rAAVs that encode cytokines either by a plasmid based-delivery system or by a recombinant virus-mediated infection has resulted in regression of tumor growth on further challenge. In a separate study, high-level IFN-γ and elevated MHC class I expression was observed after a transfer of *D122* gene-modified murine lung cancer cells that significantly delayed tumor development (142). Similar findings of antitumor immunity was reported after transfer of cytokine-encoding AAV DNA in a rat prostatic tumor model (143). Enhancements in antitumor T-cell response was observed *in vitro* by AAV-mediated transduction of *B7.1* and *B7.2* genes in a human multiple myeloma cell line (144). In a vaccination scheme, Liu *et al.* (145) have recently shown that i.m. administration of a rAAV encoding a dominant HPV16-E7 CTL epitope and a heat shock protein, delivered as a fusion protein, elicited a potent antitumor response against challenge with an E-7-expressing syngeneic cell line in immunocompetent mice. *In vitro* analysis also indicated both CD4- and CD8-dependent cytolytic activity in these studies.

AAV-based vectors have been shown to be less immunogenic when compared with other commonly used viral vectors for gene therapy. Although one of the reasons for this is the absence of vector genes in the rAAV constructs, in studies based on i.m. administration of the vector, it had been reported initially by Jooss *et al.* (19) in a mouse model that rAAV delivered by this route failed to transduce DCs, the most potent antigen-presenting cells. Reports by Brockstedt *et al.* (146), however, indicated the generation of antibody-mediated and T-cell-mediated immunity against rAAV-encoded ovalbumin delivered i.m. and i.p. Additional studies by Zhang *et al.* (147) reported that whereas mature murine DCs are refractory to AAV transduction, immature DCs are still transducible and that the transduction yields are lower in the absence of adenovirus coinfection.

Although these characteristics may limit one's ability to test rAAV in an *ex vivo* immunotherapy strategy in a murine system by genetic transfer of a potent tumor antigen gene into DCs, it may indeed be possible to evaluate the efficacy of this approach by transducing the cells before differentiation. This may, in fact, provide additional benefits such as stable expression of the AAV-transgene over time and possible integration and retention of the transgene during differentiation. The potential of such a strategy has been recently reported using human DCs *in vitro*. In these studies, transfer of the *IL-4* gene into human peripheral blood monocytes and the culturing of these cells with GM-CSF resulted in their differentiating into potent DCs (148). We have recently determined that the transfer of a rAAV that encoded the firefly luciferase in monocytes, after differentiation with IL-4 and GM-CSF, resulted in a robust increase in transgene expression in differentiated DCs (149). Using fluorescent *in situ* hybridization analysis, we were also able to identify the transgene in potent DCs 10 days after transduction (149). Similar to our earlier findings in human bone marrow-derived $CD34^+$ cells (80), we also observed differences in AAV transduction of DCs obtained from different individuals (149). Thus, advances in the development of targeted AAVs remain a priority to overcome such limitations in viral infectivity.

AAV-mediated Long-Term Cancer Gene Therapy as an Adjuvant Therapy

On the basis of several studies over the last decade concerned with cancer treatment, it is becoming increasingly apparent that gene therapy includes a repertoire of cancer treatment paradigms. At the same time, limitations in both target definition and vector efficacy need to be overcome to use this as an exclusive therapeutic modality. However, important to this discussion is the realization that gene therapy can be combined with other traditional treatments as an adjuvant therapy. For many of the solid tumors, surgery, chemotherapy, radiation therapy, and hormonal therapy constitute the major therapeutic measures. Despite advances in early detection and successful initial control, many tumors recur, yielding a much more ominous prognosis. In these situations, it may be more appropriate to advance our ability to effectively use gene therapy to prevent such recurrences. These adjunct therapies may well be targeted toward secondary cellular events such as antiangiogenesis or toward elicitation of host immunity for a greater control over local tumor recurrence or metastasis. For these strategies, rAAV remains an ideal vector because of the absence of immunogenicity and long-term/stable expression capabilities. Recent preclinical studies also indicate the feasibility of regulated expression of rAAV-transgenes *in vivo* in murine and nonhuman primate models (91–93), and it will be a next logical step to use this strategy to not only achieve high-level expression of therapeutic genes but also to do so under highly controlled conditions.

Development of Targeted AAV for Tumor-specific Delivery

Although rAAV vectors transduce both dividing and nondividing cells transcending a species barrier, it is becoming increasingly clear that there is wide variation in transduction efficiencies among different cell types (80). Recent identification of a possible cellular receptor and coreceptors for AAV (61–63) suggests that the level of expression of one or more of these molecules may determine the efficiency of infection (63). Thus, it is becoming evident that additional developments to achieve infectivity enhancements will be predicated on effective utilization of AAV-based vectors effectively in cancer gene therapy.

Recent studies have also indicated that cell-specific targets can be exploited as alternate entry pathways for AAV infection. Initial studies with targeted-AAV involved genetic and immunological modifications of vector tropism. Whereas genetic modifications of the capsid involves addition of DNA sequences representing targeting ligands (150–153), immunological modifications involve production of bispecific targeting conjugates (154). Although proven to be feasible, genetic capsid modification still requires a detailed understanding of the X-ray crystallographic structure of AAV capsid to identify ideal domains amenable for alterations. By mutational analysis, recent studies have also identified potential regions in the AAV capsid that may be used in genetic modifications (151–153). Despite these possibilities, an additional concern with AAV is the size of the capsid molecule. Because AAV is a small virus of ~25 nm, genetically adding larger sequences may drastically impair the virus assembly, titer, and infectivity. Thus, it is also important to identify more cancer cell-specific ligands and characterize their binding epitopes to be used in targeting strategies.

By using bispecific antibody conjugates involving fibroblast growth factor, Bartlett *et al.* (154) reported the feasibility of immunological targeting of M07e cells, which are otherwise refractory to AAV infection. Although effective, such an immunological targeting requires large amounts of purified antibodies. Furthermore, the *in vivo* stability of chemically conjugated antibodies may limit their potential application in cancer gene therapy. Thus, further development of targeting AAV that can achieve high-efficiency production and stability of the vector will aid in future cancer gene therapy applications. Lastly, it is also important to ablate native tropism of the vector for targeted delivery because retention of epitope(s) in the vector capsid that interact with the native receptor may result in the transduction of nontarget cells. Considering the fact that AAV infects many cell types, this may be a crucial requirement in optimal utility of targeted AAV. In addition to transductional targeting, construction of rAAV that can achieve transcriptional targeting may also benefit AAV-mediated cancer gene therapy applications. The recently completed human genome analysis and the technological advances, including the powerful microarray and proteomics, are increasingly able to molecularly dissect subtle differences in tissue-specific expression and are rapidly being exploited in cancer research. Information derived from such technological advancement should aid in the design of ideal AAV vectors for transductional and transcriptional targeting in future cancer gene therapy applications.

Conclusions

On the basis of multiple studies over the last several years, it is becoming increasingly clear that rAAV vectors are potential alternatives to other viral vectors for gene therapy. Although a majority of preclinical studies with rAAV have historically centered around correction of genetic and metabolic diseases, recent studies indicate the potential of AAV vectors in cancer gene therapy. It is also becoming

apparent that for genetic therapy for cancer to be successful, a wide spectrum of target molecules and cells may be effectively used. The salient features of AAV, such as long-term expression, potential of high-efficiency transduction, low host immunity, and native tumor suppressor properties, succinctly reviewed in this article, suggests that these properties can be wisely exploited in therapeutic and preventive cancer gene therapy strategies. Additional advances in the basic biology of the vector should lead to the development of second-generation, high-efficiency and cell-specific vectors, which in turn, will lead to the emergence of novel vector paradigms advancing future cancer gene therapy applications.

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