Gene Therapy Applications of Viral Vectors

Viral vectors have frequently been applied in gene therapy with the final goal of treating various diseases in the areas of neurology, neurodegeneration, metabolic disease, and cancer. Vectors have been engineered based on AAV, adenoviruses, alphaviruses, herpes simplex viruses, lentiviruses, and retroviruses. Some vectors are suitable for short-term episomal transgene expression, whereas others are integrated into the host cell genome to provide long-term expression. Additionally, hybrid vectors with favorable features from different viruses have been developed. Therapeutic genes of choice have typically been toxic genes such as thymidine kinase, pro-apoptotic genes like Bax, and immunostimulatory genes (for instance, interleukin-12). A large number of animal studies have demonstrated proof of concept of viral gene therapy. Many types of viral vectors have been employed in more than 700 clinical trials that have been carried out or are currently in progress.

Introduction

As of today, there are approximately 1000 clinical trials conducted or in progress applying gene therapy applications (1). The majority of these, almost 70%, are based on viral vectors. Two important features of viruses have made them attractive to use. Viral vectors have generally proved efficient vehicles for gene delivery to target cells/tissue, a critical aspect of achieving therapeutic efficacy. Another important factor has been the establishment of high level transgene expression. Viruses typically possess strong promoters, which can generate high level gene expression in infected host cells. However, there is a wide repertoire of viral vectors with highly different properties. For instance, some vectors like adenoviruses and alphaviruses are of an episomal nature and therefore provide for a rather limited duration of transgene expression. Especially alphaviruses carrying a single-stranded RNA genome, because they generally allow a rapid onset of transgene expression, but only for 7-10 days at very high levels (2). In contrast, adeno-associated virus vectors and retrovirus vectors have the capacity to integrate into the host genome, which therefore provides really long-term expression. However, the drawback, especially for retroviruses, is the random integration that can potentially activate oncogenes as summarized in this review. Herpes simplex viruses have a feature of latency, which can provide a life-long co-existence with the host organism. Obviously, the choice of short- or long-term transgene expression is strongly dictated by the targeted disease. For instance, neurodegenerative and metabolic diseases characterized by a non-existent or deficient production of an enzyme requires long-term functional expression of the healthy gene. However, in treatment of cancer with viral vectors expressing highly toxic or therapeutic proteins with severe side effects, the preference is a transient nature of expression.

In this review, highlights are presented for the main types of viral vectors that have been applied in gene therapy today. Examples of novel applications are
described. Also the possibility to combine favorable features of different viruses in so-called hybrid vectors is discussed. Finally, a brief overview of safety aspects and recent developments in clinical trials with viral vectors is included.

**Adenoviruses**

Adenoviruses are, in addition to retroviruses, the most applied viral vectors in gene therapy. These double-stranded DNA vectors have the capacity to harbor approximately 8 kb of foreign DNA and are efficiently packaged in mammalian cell lines such as HEK3 cells (3). Adenoviruses demonstrate a relatively broad host range and can infect both dividing and non-dividing cells. Due to their episomal nature, their expression pattern is transient; although in certain cases, long-term expression has been observed. For instance, a recently developed canine adenovirus vector generated reporter gene expression for a year in the rat cortex (4). The expression was highly neuron-specific with green fluorescence protein (GFP) detected in striatal and cholinergic neurons. Despite the broad host range of adenovirus vectors, the success of transducing hematopoietic cells has been rather modest. Hybrid adenovirus vectors with the cell receptor recognition fiber from the adenovirus 35 serotype and the capsid from Ad5 were capable of significantly improved transduction rates of hematopoietic cells (5). An oncolytic adenovirus vector with fibers from Ad35 and the E1a gene for specific replication in tumor cells only was engineered to express the TNF-related apoptosis inducing ligand (TRAIL) gene (6). Existing liver metastasis were efficiently eliminated after tail vein infusions with this adenovirus vector. An interesting approach of re-targeting the adenovirus vector was obtained by conjugation of polyethylene glycol (PEG) to the adenovirus capsid (7). The PEGylated adenovirus showed targeting to endothelial cells and a 12-fold longer duration in the circulation.

To generate an adenovirus capable of replication in only uterine cervical cancer cells, a vector with the MN/CA9 promoter was engineered (8). As the MN/CA9 glycoprotein is highly expressed in cancer cells but not in normal cells, application of the Ad-MN/CA9-E1a vector resulted in strong inhibition of MN/CA9 cancer cell growth and HeLa tumor growth.

### Table I

Properties of various viral vectors and their applications in gene therapy.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Host range</th>
<th>Features</th>
<th>Applications</th>
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<tbody>
<tr>
<td>AAV</td>
<td>relatively broad</td>
<td>slow onset of expression integration into chromosome 19</td>
<td>atherosclerosis, hemophilia, colorectal cancer</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>relatively broad</td>
<td>episomal short-term expression immunogenicity</td>
<td>various cancers, metabolic diseases, CNS</td>
</tr>
<tr>
<td>Alphaviruses</td>
<td>broad</td>
<td>short-term, extreme expression neuron and glial specific strains low immunogenicity</td>
<td>cancer vaccines, melanoma &amp; kidney carcinoma, CNS</td>
</tr>
<tr>
<td>Baculovirus</td>
<td>restricted</td>
<td>transient expression extreme MOIs</td>
<td>cardiovascular delivery, brain delivery</td>
</tr>
<tr>
<td>Herpes viruses</td>
<td>broad</td>
<td>latent infection long-term expression low toxicity (mutants)</td>
<td>neurodegeneration, cancer, CNS, PNS</td>
</tr>
<tr>
<td>Lentiviruses</td>
<td>broad</td>
<td>chromosomal integration long-term expression safety concerns inefficient virus production</td>
<td>HIV, cancer, neurodegeneration, CNS</td>
</tr>
<tr>
<td>Poxviruses</td>
<td>broad</td>
<td>transient expression low immunogenicity</td>
<td>cancer vaccines, prostate cancer</td>
</tr>
<tr>
<td>Reoviruses</td>
<td>restricted</td>
<td>replication in tumor cells toxicity</td>
<td>cancer</td>
</tr>
<tr>
<td>Retroviruses</td>
<td>restricted</td>
<td>genome integration (random!) long-term expression</td>
<td>SCID-X1 cancer, cancer</td>
</tr>
<tr>
<td>SV40</td>
<td>limited broad</td>
<td>high-titer virus production low immunogenicity chromosomal integration</td>
<td>cancer, liver delivery, hepatitis</td>
</tr>
</tbody>
</table>

CNS, Central nervous system; MOI, multiplicity of infection; PNS, peripheral nervous system.
delay in nude mice after intratumoral injections. Another adenovector capable of conditional replication in p53-deficient human tumor cells only demonstrated GFP-zeosin fusion protein expression in glioblastoma and bladder cancer cells, but not in normal fibroblasts with a functional p53 expression (9).

A novel replicative adenovirus vector CNHK300, in which the human telomerase reverse transcriptase (hTERT) promoter was introduced, was evaluated both in vitro and in vivo (10). The three E-boxes introduced downstream of the promoter decreased the promoter activity in normal cells but not in cancer cells verified by luciferase activity. Studies with the adenoviral E1a gene as a therapeutic gene demonstrated selective replication and oncolytic and anti-tumoral effects. In another study, the adenoviral E1a promoter was replaced by the human tyrosinase enhancer/promoter to engineer a conditionally replication-competent adenovirus vector (11). The transduction rate in melanoma cells was improved by the introduction of an RGD-4C peptide into the fiber knob of the Ad.Tyr-E1a vector. Tumor regression was observed in mice, which led to normalization of the serum lipid levels and expression levels of factor IX (FIX) in liver cells than any serotype. The AAV7 and AAV8 serotypes, isolated from rhesus monkeys, have demonstrated 100-fold higher transgene expression levels of factor IX (FIX) in liver cells than any other AAV serotype (15). The AAV7 and AAV8 vectors were also expressed in the portal vein of LDL receptor-deficient mice, which led to normalization of the serum lipid levels and protection against development of severe atherosclerosis (16).

Several serotypes of AAV exist, each showing substantial differences in their properties of cell transduction and transgene expression patterns. For instance, the AAV1 serotype is suitable for expression in skeletal muscle and retina cells, whereas the AAV2 shows more moderate but long-term expression. The AAV5 serotype generates high expression levels in neuronal and lung cells. As immune responses have been obtained upon re-administration of AAV particles, one solution has been to package the therapeutic gene in another AAV serotype. The AAV7 and AAV8 serotypes, isolated from rhesus monkeys, have demonstrated 100-fold higher transgene expression levels of factor IX (FIX) in liver cells than any other AAV serotype (15). The AAV7 and AAV8 vectors were also expressed in the portal vein of LDL receptor-deficient mice, which led to normalization of the serum lipid levels and protection against development of severe atherosclerosis (16).

The long-term effect of AAV expression was demonstrated by injection of AAV particles expressing the truncated solubly form of the vascular endothelial growth factor receptor-2

### Adeno-Associated Viruses (AAV)

AAV vectors have become increasingly popular for gene therapy applications as the methodology for high-titer virus production has developed. Originally, the methods were not applicable to large scale production, but recent developments such as using baculovirus vectors for AAV production in insect cells (13) has made them compatible. The AAV particles produced in insect cells showed the same physical and biological properties as those produced in mammalian cells with a much improved efficacy (the yield from a one liter insect cell culture was equivalent to 500-1000 T-flasks of mammalian cells). The small size of AAV allows insertion of only a restricted length (<4 kb) of foreign DNA. The integration of AAV into the host genome generally occurs in a defined area of chromosome 19, although random integration has also been described. The chromosomal integration provides a means of long-term transgene expression, which has been observed in many different tissues such as liver, muscle, retina, and the central nervous system (14).

Figure 1: Schematic presentation of viral vectors and the time required. The various steps involved in preparation of virus stocks are briefly described. The time required for the preparation does not include subcloning into vectors and preparation of plasmid DNA. However, these procedures are generally independent of which viral vector system is used.
(VEGFR-2) in the portal vein of mice (17). As the AAV vector also contained the fetal liver kinase-1 gene (flk-1), expression was confirmed by ELISA. Neuroblastoma tumors were induced in the animals after 8 weeks. The long-term AAV-based expression of a functional VEGF inhibitor clearly demonstrated anti-angiogenic and anti-cancer effects in mice. AAV vectors have also been applied in a mouse model of lysosomal storage disease (18). In this study, $10^{10}$ AAV particles expressing the human iduronidase (IDUA) gene were intravenously injected, which resulted in long-term (5 month duration of the study) expression of IDUA, preferentially in heart and lung. Animals treated with the AAV-IDUA vector showed histopathological reduction in lysosomal storage in several tissues and had a curative impact on the most important parameters of the disease.

**Alphaviruses**

Alphaviruses have been frequently used as expression vectors for recombinant protein production (19) and as vaccine vehicles (20). The most commonly used alphavirus expression systems are based on Semliki Forest virus (SFV) (21), Sindbis virus (SIN) (22) and Venezuelan equine encephalitis virus (VEE) (23). Alphavirus vectors are generally replication-deficient and can be applied as nucleic acid vectors (DNA plasmid or naked RNA) and recombinant particles. High-titer (up to $10^{10}$ infectious particles per milliliter) can be generated in less than two days, and their broad host range makes them applicable to many types of cell lines, primary cells and in vivo studies. The transient nature of alphavirus vectors more or less eliminates their use in chronic gene therapy applications. In contrast, this feature makes them attractive for cancer gene therapy, where short-term and high-level gene expression is desirable.

Cancer vaccine production is an area where alphavirus vectors have been frequently applied. For instance, the immunization of animals with recombinant SFV particles expressing tumor antigens such as P185 has provided protection against tumor challenges (24). DNA-based alphavirus vectors have also been used for immunization, but their efficacy has generally been lower than recombinant particles, although their safety level is higher. Furthermore, immunization with SFV-LacZ RNA resulted in tumor regression in mice (25). Another approach has been to vaccinate animals with dendritic cells previously pulsed with SFV particles expressing interleukin-12 (IL-12), which resulted in tumor regression in a glioma mouse model (26).

The efficacy of intratumoral SFV injections was demonstrated in a B16 melanoma model (27). Administration of SFV particles expressing the p40 and p35 subunits of IL-12 showed tumor regression and inhibition of tumor blood vessel formation measured by Doppler ultrasonography. In another study, intratumoral injections of SFV-GFP virus led to substantial tumor shrinkage in a mouse model implanted with human lung tumors (28). The immunodeficient mice showed the best response after 3 injections on consecutive days followed by another 3 injections one week later. The growth of AT3-Neo and AT3-Bcl2 tumors was also reduced in nude mice after intratumoral administration of SFV vectors expressing the pro-apoptotic Bax gene (29). Systemic delivery of alphavirus vectors has, however, been of some concern due to the strong preference of expression in neuronal cells in vivo (2). Attempts have therefore been made to target the expression to specific cells/tissue by the introduction of targeting sequences in the envelope of SIN virus. To demonstrate the proof of principle, IgG binding domains of protein A were introduced into the E2 envelope protein of SIN, which resulted in chimeric vectors with a significantly modified host range (30). These vectors showed a 10-fold reduction in transduction rates of BHK and other susceptible cell lines, but could efficiently infect cells treated with a monoclonal antibody against a surface protein. Quite surprisingly, a recent study with a conventional unmodified SIN vector demonstrated significant targeting to various tumors after systemic delivery (31).

Another approach has been to encapsulate SFV particles in liposomes that are targeted to tumor cells. Encapsulation of SFV-LacZ particles resulted in efficient tumor targeting and a strong expression of β-galactosidase in human LNCaP prostate tumors that are implanted in SCID mice, with only minor staining of the normal tissue in SCID mice (32). Tumor growth inhibition was demonstrated in mice with pancreatic tumors after systemic delivery of liposome-encapsulated SFV-IL-12 particles (33). Moreover, a preliminary clinical phase I trial showed safe delivery and 5-fold elevated levels of IL-12 in the serum of kidney carcinoma and melanoma patients after administration of encapsulated SFV-IL-12 particles (32). The results of this trial have, however, not been published yet and are just briefly mentioned in a review on liposomal encapsulation technologies (32).

**Herpes Viruses**

Herpes simplex viruses (HSV) have several features that have made them attractive as gene therapy vectors. Their large 152 kb DNA genome allows for the introduction of large foreign DNA insertions and permits modifications, including deletions of non-essential genes such as ICP0, ICP4, ICP22, and ICP47, which has generated vectors with substantially reduced cytotoxicity (34). Moreover, although HSV vectors transduce many cell types they have a strong neurotropism, which should make them suitable for treatment of neurodegenerative diseases. They can also establish a latent life-cycle, which means that they can present a life-long persistence in host organisms, and therefore provide long-term transgene expression.
The engineering of HSV vectors with the latency active promoters LAP1 and LAP2 resulted in really long-term transgene expression (>1 year) in the peripheral nervous system (35). In another study, a conditionally replicating HSV vector expressing prepro-encephalin was able to release functional encephalin in the PNS (36). In an attempt to treat neuropathic pain after spinal cord injury, an HSV vector carrying the human glutamic acid decarboxylase gene GAD67 was subcutaneously inoculated into both feet of Sprague-Dawley rats. The HSV-mediated GAD67 expression in dorsal root ganglion neurons suggested that neuropathic pain could be treated after incomplete spinal cord injury by this approach (37). HSV vectors have also been used for expression of interleukins in the brain. When mice were treated with HSV-IL-4, the levels of Th2 cytokines (IL-4 and IL-10) were elevated, whereas the type TH1 cytokine IL-23 showed similar levels as in control animals (38). Surprisingly, HSV-IL-10 treatment resulted in a reduction of IL-23 expression. However, for gene therapy applications the favorable effect of IL-4 expression is more important than the downregulation of Th1 type cytokines.

As for other vectors, replication-competent HSV vectors which can replicate and spread only in tumors and not in normal tissue have been developed (39). Oncolytic HSV vectors have been evaluated in many different animal tumor models including glioma, melanoma, breast, prostate, colon, ovarian, and pancreatic cancer (40). Improved efficacy was obtained by combination treatment with radiotherapy or chemotherapy. A mutant HSV vector with deletions in the γ(1) 34.5 gene demonstrated eradication of tumors and improved long-term survival in SCID mice with intracranial gliomas (41). Application of another oncolytic HSV vector expressing the GFP reporter gene, NV1066, resulted in specific replication and strong cytotoxicity in OCUM gastric cancer cells (42). The GFP expression in OCUM cells was dose-dependent, but interestingly, infection with very low virus concentrations (multiplicity of infection of 0.01) led to almost complete lysis of the cells after 7 days. The GFP expression was also prominent in vivo in macroscopic tumor foci, but not in normal non-tumor cells.

**Retroviruses**

Early gene therapy studies were to a large extent based on retrovirus vectors, typically applying murine leukemia virus (MLV). Retroviruses are double-stranded RNA viruses possessing reverse transcriptase activity, which enables them to integrate as DNA copies in the host genome (43). Due to the chromosomal integration long-term transgene expression can be established. Retroviruses show a relatively broad host range although non-dividing cells are not susceptible. In this context, retrovirus vectors have been considered attractive for the treatment of brain tumors as the neuronal cells are not affected. For instance, experiments in cell cultures and animal models have demonstrated efficient retrovirus-mediated gene transfer and killing of glioma cells (44). In a study on retrovirus vectors, the growth arrest specific 1 (Gas1) gene was expressed in C6 glioma cells applying the human glial fibrillary acidic protein 2 (gfa2) promoter (45). Retrovirus transduction induced apoptosis in C6 glioma cells and significantly reduced their viability. An inhibition of tumor growth was observed in nude mice implanted with gliomas. Replication-deficient retrovirus vectors have also been applied for studies on pancreatic cancer. When the GINaTK retrovirus vector with the HSV-TK gene was transduced into pancreatic cancer cells, efficient cell killing and a remarkable bystander effect was observed after ganciclovir treatment (46).

The most exciting and impressive gene therapy application of retrovirus vectors so far has definitely been the treatment of infants with the SCID-X1 (severe combined immunodeficiency) disease (47). The immune deficiency is caused by a mutation in the γc gene and was corrected by infusion of hematopoietic stem cells ex vivo transduced with a retrovirus vector carrying the healthy gene. The results were encouraging, with 9 out of 10 patients cured. Unfortunately, a T-cell leukemia developed in 2 patients after 3 years (48). This unexpected outcome was due to the integration of the therapeutic gene in an oncogenic region of the chromosome, discussed in more detail in the section on safety. As a consequence of this finding, more efforts will be dedicated to the development of novel retrovirus vectors with targeted integration. A modified vector MND-IL-2R has been developed for SCID-X1, which contains the IL-2 γcDNA and the Moloney murine leukemia virus (MoMLV) enhancer substituted with the corresponding region from the myeloproliferative sarcoma virus (MPSV) (49). The MND-IL-2R vector demonstrated efficient transduction of human CD34+ progenitor cells.

**Lentiviruses**

Lentiviruses are also retroviruses, but have some unique features that give them a broader application range in gene therapy. They share many common features with retroviruses such as the dsRNA genome, relatively good capacity of packaging foreign genes and the means to integrate into the host genome (50). However, different from other retroviruses, lentiviruses can efficiently transduce both dividing and non-dividing cells, which make them attractive for applications in neurobiology too.

Lentivirus-based delivery of the glial cell line-derived neurotrophic factor (GDNF) has been evaluated in a primate model for Parkinson’s disease (51). Lent-GDNF virus injected into the striatum and substantia nigra of aged rhesus monkeys treated with 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine (MPTP) reversed the dopaminergic functional deficits.
Lentiviruses have also been used for RNA interference (RNAi) approaches. BRAF mutations are found in 66% of melanomas and lentivirus-based RNAi vectors specific for the wildtype and the most frequently mutated BRAF (V599E) were evaluated in 10 melanoma cell lines (54). The HIV-siRNA for BRAF V599E inhibited the growth of melanoma cell lines and lentivirus-based RNAi could therefore be a powerful tool for targeting oncogenic mutations in the future. An interesting application of lentivirus-based gene therapy was the induction of long-term tolerance of 1,3 galactosyltransferase deficient heart grafts (55). Bone marrow from gal knockout mice was transduced with a lentivirus vector expressing the porcine 1,3 galactosyltransferase and transplanted into lethally irradiated gal knockout mice. Hearts from wildtype gal donors were permanently accepted and demonstrated that lentivirus can establish stable chimerism and induce long-term heart graft tolerance.

Reoviruses

Reoviruses have become potentially interesting cancer therapy vectors because of their preferential infection and killing of cells with an active Ras signalling pathway (56). The oncolytic nature of reoviruses renders them directly applicable to cancer treatment without the presence of any toxic or therapeutic foreign gene. Reovirus killed 20 out of 24 established cancer cell lines and all 9 tested glioma biopsies from patients (57). In vivo, complete tumor regression was observed in mice with subcutaneous and intracerebral human glioma xenografts after injection of live reovirus. Studies in breast tumor-derived cell lines and biopsies from cancer patients demonstrated cell killing by reoviruses (58). Likewise, the cytopathic effect of reoviruses was showed in several human colon and ovarian cancer cell lines, but not in normal colon and ovarian cell lines (59).

SV40

The high-titer SV40 virus production, the broad host range and the relatively weak immune response against SV40 have made them potentially attractive as gene therapy vectors (60). One drawback of SV40 is the limited packaging capacity of foreign DNA. The main applications of SV40 gene transfer have been on hematopoietic progenitor cells such as CD34+ cells and liver cells. SV40 vectors have also been studied in relation to hepatitis B (HBV) infection. The SV40 transduction rate of human hepatocytes was significantly higher in the HBV positive cell lines HepG2.2.15 and DLC4-A10II than in control HepG2 cells based on luciferase expression (61). Furthermore, the SV40 DNA concentration in the nuclei of HepG2.2.2.15 cells was 6-fold higher than in control HepG2 cells. For this reason, SV40 vectors could find therapeutic use in the treatment of HBV infections.

Vaccinia

Vaccinia viruses belong to the family of poxviruses, which are large dsDNA viruses that in contrast to other DNA viruses replicate in the cytoplasm. Both non-replicating and replication-competent vaccinia virus expression vectors have been engineered (62). Deletions in the thymidine kinase (TK) gene and the vaccinia growth factor (VGF) gene resulted in a mutant vaccinia vector, which showed a high replication rate in tumor cells, but not in normal cells (63). Systemic delivery of the mutant vaccinia vector in mice with subcutaneous tumors was safe and the treatment was successful.

Recombinant vaccinia virus vectors have also been used in cytokine immunotherapy. Significant tumor growth inhibition was observed in nude mice after a single intratumoral injection of vaccinia virus expressing either interleukin-2 (IL-2) or IL-12 (64). The prolonged survival of the animals was due to the immune responses induced by IL-2 and IL-12. In a C6 glioma mouse model, doses from 10^5 to 10^7 plaque forming units (PFU) of an attenuated vaccinia-IL-2 or rIL-12 vector resulted in an anti-tumor response, which was not dose dependent (65). However, the toxicity was much higher for doses of more than 10^5 PFU. A correlation was found between the anti-tumor activity, the number of natural killer T cells in the spleen and the local induction of interferon-γ and TNF-α responses.

Baculovirus

Baculovirus vectors have generally been associated with high-level recombinant protein expression in insect cells (66). Modifications to the baculovirus expression system and the introduction of the BacMam vectors have made it possible to use these insect vectors for infection and overexpression of recombinant proteins in mammalian cells too (67). As infected human cells cannot support baculovirus replication in human cells the use of these vectors for gene therapy applications in humans was deemed safe.
One drawback of applying baculovirus vectors for transduction of mammalian cells is the need of relatively high multiplicity of infection (MOI). Despite this, in vivo gene delivery studies with baculovirus vectors have been conducted in both rabbit carotid artery (68) and rat brain (69). Rabbit aortic smooth muscle cells as well as human ECV-304 cells were susceptible to baculovirus-mediated β-galactosidase expression (70). In comparison to adenovirus-based gene delivery in rabbit carotid arteries, 10^9 PFU of baculovirus, generated similar expression levels of β-galactosidase. The expression was located at the adventitial cells in the carotid arteries and lasted for approximately 1 week. In the rat brain, baculovirus specifically transduced the cuboid epithelium of the choroid plexus in the ventricles (71). The expression levels were comparable to adenovirus, but the response in microglia was only modest whereas adenovirus induced a strong response.

**Hybrid Vectors**

The engineering of hybrid vectors has allowed the combination of favorable features from different viral vectors. A chimeric adenovirus-AAV vector containing a first generation adenovirus vector and a reporter gene flanked by the inverted terminal repeat (ITR) sequences of AAV was constructed to achieve chromosomal integration (72). Stable long-term expression was demonstrated for a randomly integrated reporter gene in a transduced cell line.

A hybrid vector between an adenovirus and a retrovirus has also been constructed by introduction of the MoMLV long terminal repeats flanked by two loxP recognition sites into an adenovirus vector. Although the natural intermediate for retroviral integrase is linear DNA, the excised circular DNA could be integrated into the host cell genome in the presence of retroviral Gag and Pol (73). The generation of high-titer adenovirus-mediated delivery of retroviral provirus with long-term (>3 months) survival of clones became possible after the introduction of a selectable marker in the vector. The transduction frequency of hybrid vectors could be enhanced by co-transduction of adenoviruses carrying the retroviral genome and the GFP reporter gene (AxC2.GCEGFP) and the retroviral packaging protein genes (AxTetGP), respectively (72). Complete tumor regression in 50% of established subcutaneous 9L tumors were observed in nude mice after co-injection of AVC2.GCTK and AxTetGP vectors. In another study, stable transgene expression in 70% of the transduced cells was achieved for a hybrid vector between an adenovirus and a foamy virus, a subfamily of retroviruses (73).

Hybrid vectors have also been engineered between HSV and AAV, which allowed site-specific chromosomal integration. Introduction of AAV inverted terminal repeats (ITRs) into an HSV-1 amplicon backbone resulted in the rep(-) HSV/AAV vector, whereas the rep(+) HSV/AAV hybrid had the rep68/78 outside the 3′ end AAV ITR (74). The rep(+)hybrid vector; but not the rep(-)vector, showed increased transduction rates in human 293 and Gli36 cell lines and in primary myoblasts. The integration was site-specific, 84% into the AAVS1 site of Gli36 stable clones and in 40% of 293 cells. It has also been demonstrated that the stable integration frequency was 10-fold higher in fibroblasts with the rep(+) HSV/AAV vector than with the rep(-) vector (75). Moreover, the combination of an HSV-Epstein-Barr hybrid vector with retrovirus vector sequences generated a trivirion vector that was able to convert tumor cells into retrovirus producer cells (76). This approach produced a local 4-fold amplification of stable transgene expression in tumors and demonstrated that transgene cassettes can be integrated into tumors.

**Safety Aspects**

The vast number of preclinical studies in cell lines and animal models should ensure the highest level of safety for studies in humans. Despite this and the demanding and detailed information required before permission is granted for any clinical trials with gene therapy vectors, some cases of severe adverse events have been reported. Obviously, the most severe case is the fatal systemic inflammatory response syndrome described in an ornithine transcarbamylase-deficient patient treated with an adenovirus vector (77). Although it is most regretful that such an event occurred related to this adenovirus gene therapy trial, it had a positive effect on applying better protocols and further engineered vectors as means of improved safety.

Another case of severe adverse events was reported for the treatment of infants with a SCID-X1 phenotype with a retrovirus vector (47) (described in the section on retroviruses). The excitement of the excellent success rate of 9 out of 10 cured patients was dampened by the findings that leukemia had developed in two of the patients, nearly three years after the treatment (48). The random chromosomal integration was determined as the cause and a closer analysis of the integration site suggested an accumulation in the LMO-2 oncoprotein (78). More detailed studies, however, indicated that both the therapeutic gene and the gene at the insertion site are potential oncoproteins resulting in an exceptionally high synergistic oncogetic effect (79). Again, the lesson learned is that more research is required to study the mechanism of chromosomal integration and to develop technologies for safe site-specific insertion into the genome.

The risks of AAV vectors applied in gene therapy have been thoroughly evaluated (80). In this context, it was demonstrated that the site-specific integration into chromosome 19 occurred in 60% of latently infected cell lines and in general only 1 out of 1000 infectious units could integrate. Recombinant AAV forms predominantly episomal concate-
mers, but their risk of causing insertional mutagenesis and oncogene activation is considered as low. Another point of concern has been the findings of low transient amounts of AAV DNA in body fluids and distal organs. For instance recombinant AAV sequences have been found in the urine, saliva and the serum of patients. After AAV delivery to the hepatic artery some transient shedding into the semen was also observed. However, germ cells do not seem to be susceptible to AAV transduction, and therefore the risk of transfer of new genetic material into the germ line is absent or extremely low. Finally, gene therapy applications of HIV-based lentivirus vectors still suffer from the psychological barrier related to the use of a known pathogen in humans. However, the state-of-the-art lentivirus vectors present a minimal risk for human applications (81) and the first clinical trial is now in progress applying lentiviral vectors in HIV-infected patients (82).

**Clinical Trials**

Since 1990, the number of clinical trials using viral vectors increased every year until 1999, when the first severe adverse events were reported (77). This incidence sparked the regulatory agencies to put several trials, at least temporarily, on hold. The set backs in the SCID-X1 trial with retrovirus vectors in 2002 (48) had a further negative impact on gene therapy trials. Among current and terminated trials, retroviruses are the most frequently used (28%), closely followed by adenoviruses (26%) (1). Poxviruses represent (3.3%), herpes simplex viruses (2.8%) and AAV (2.1%) of the trials.

The cure of 9 out of 10 infant SCID-X1 patients with a retrovirus vector must still be considered the most impressive clinical gene therapy application, so far (47). Some highlights on other clinical trials in progress are summarized below. In a phase I trial on patients with advanced pancreatic, colorectal and primary liver tumors, adenoviral vectors expressing IL-12 were administered intratumorally (83). Ad-IL12 was administered 3 times per month at doses of $2.5 \times 10^{10}$ to $3 \times 10^{12}$ virus particles. The treatment was well tolerated and adverse reactions observed such as fever, malaise, sweating and lymphopenia were concluded to be related to the vector and not to the transgene expression. A stable disease was obtained in 29% of the patients.

AAV vectors have been applied in two clinical trials for hemophilia B, a deficiency in factor IX (FIX) (84). Previously, the canine FIX was expressed from an AAV vector at therapeutic levels for an extended period in a hemophilia B dog model (85). In one of the studies, AAV-FIX was introduced intramuscularly into the upper and lower extremities of 8 patients and long-term FIX expression was demonstrated by Southern blots and immunofluorescence in muscle biopsies taken 2 and 10 months post-injection. Based on animal studies, the number of required injections to reach the therapeutic levels in humans were, however, not practically feasible. Despite that, the safe delivery and long-term expression of FIX in humans was demonstrated. The other AAV-FIX trial on systemic administration to target the liver is still in progress with 6 patients enrolled.

Vaccinia virus vectors have been applied for a few cancer therapy trials. The prostate-specific antigen (PSA) was expressed from vaccinia virus vectors in a phase I study on patients with advanced prostate cancer (86). At 3 consecutive monthly doses of $2.65 \times 10^{10}$, $2.65 \times 10^{11}$ and $2.65 \times 10^{12}$ PFU no virus-related adverse effects were detected. The PSA levels in 14 out of 33 patients were stable for at least 6 months and 9 patients remained stable for 11-25 months. At the end of the study, 6 patients were still progression free. In another study, a replication-restricted vaccinia virus vector was used for intratumoral injections of vaccinia virus particles expressing IL-2 (10^{7} PFU per dose for 12 weeks) in 6 patients with treatment-resistant mesothelioma tumors (87). The treatment showed minimal toxicity and the expression of IL-2 lasted for up to 3 weeks. In a study on metastatic breast cancer, 14 patients received repeated intramuscular injections (at doses of $5 \times 10^{6}$ and $5 \times 10^{7}$ PFU) of an attenuated vaccinia virus TG1031 expressing the human MUC1 gene and the IL-2 gene. Partial tumor regression (>50%) was observed in 2 patients and stable disease in 15 patients (88).

In summary, the last decade has seen major improvement in vector development and the collection of a large number of different types of vectors, which should suite different areas of applications. Clinical trials have also showed some encouraging results. However, there is still a vast amount of research and development in front of us before gene therapy can fulfill the promise as the medicine of the future.

**Reference**

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Gene Therapy Applications of Viral Vectors


Date Received: July 16, 2004