

Development and use of viral vectors for gene transfer: lessons from their applications in gene therapy

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Abstract: Gene therapy possesses great potential to revolutionise the clinical management of many human diseases. With the completion of the human genome project, it is anticipated that significant progress will be made in identifying disease-causing genes or genes with therapeutic potential. Since the first clinical trial in 1990, over 470 clinical gene therapy protocols have been approved in the United States. A valuable lesson learned in this first decade is that the efficacy of gene therapy is profoundly affected by the efficiency of delivery and appropriate expression of therapeutic genes. As naturally evolved vehicles for gene transfer, viral vectors are popular choices in gene therapy. Currently, viral vectors derived from retroviruses, adenoviruses, adeno-associated viruses and the herpes simplex virus have been used in more than two-thirds of clinical gene therapy trials worldwide, and most viral vectors have demonstrated an acceptable biosafety profile. Retroviral vectors are used when long-term expression of a therapeutic gene is required. Their use has been hampered by their inability to infect non-dividing cells, although this caveat can be circumvented by the recently developed lentiviral vectors. Conversely, adenoviruses and adeno-associated viruses are used when transient expression of a transgene is desired. They can transduce both dividing and non-dividing cells with high efficiency. However, adenoviral vectors tend to elicit a significant host immune response. Recently, herpes simplex viral vectors have emerged as an alternative delivery vehicle, especially for gene transfer to central and peripheral nervous systems, although a significant improvement of their potential pathogenic effect is required. Therefore, the use of each vector should be largely determined by the virology of an individual vector and the purpose of gene transfer. It is anticipated that significant progress will be made in refining current vectors and in developing new viral vectors during the second decade of gene therapy. Here, we review the current state of human gene therapy by focusing on the development and application of viral vectors as gene transfer vehicles.

Keywords: adenovirus, adeno-associated virus, gene therapy, herpes simplex virus, lentivirus, retrovirus, viral vectors

Current state of human gene therapy

Introduction

By using directed gene transfer to treat human disease, gene therapy may hold the potential to revolutionise medicine – in part because this approach is capable of treating the root cause of a disease, not merely its symptoms. The first clinical gene therapy study was initiated in 1990 (Rosenberg et al 1990) and investigations have steadily grown since then. Results from early clinical trials were disappointing, largely because the available vectors for gene transfer were inadequate (Wadman 1995). In fact, the lack of data on the efficacy of viral vectors in most clinical trials prompted the National Institutes of Health of the United States (NIH) to appoint an expert committee in 1995. The committee eventually recommended that resources be diverted away from premature clinical trials with ineffective vectors and

applied to basic studies on gene transfer and gene expression technologies that are more relevant to pre-clinical testing. This decision led to more productive investigations into vector development and improved gene transfer efficiency. With the completion of the human genome project and the identification of more disease-causing genes, research into gene therapy will continue to expand at a dramatic pace.

Since 1990, over 470 gene therapy protocols have been approved by or submitted to the NIH (<http://www4.od.nih.gov/oba>). Thus far, all clinical protocols have involved gene transfer to exclusively somatic cells, as

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Table 1 Summary of gene therapy protocols approved by or submitted to the National Institutes of Health of the United States

<i>Classified by disease</i>	<i># of protocols</i>	<i>% of protocols</i>
Cancer	326	69.1
AIDS/HIV infection	36	7.6
Genetic diseases	29	6.1
Cardiovascular diseases	44	9.3
Rheumatoid arthritis	2	0.4
Multiple sclerosis	1	0.2
Acute hepatic failure	1	0.2
Others	33	7
Total	472	100

<i>Classified by vector</i>	<i># of protocols</i>	<i>% of protocols</i>
Retrovirus	168	35.5
Adenovirus	138	29.3
Adeno-associated virus	14	3
Vaccinia virus	33	7
Herpes simplex virus	2	0.4
Non-viral	117	24.8

<i>Classified by delivery route</i>	<i># of protocols</i>	<i>% of protocols</i>
In vivo	284	60.2
Ex vivo	188	39.8

Source: <http://www4.od.nih.gov/oba>

opposed to germ-line cells, although this may change with the US government's recent decision to allow research involving certain stem cell lines. Somatic gene therapy can be carried out either *ex vivo* or *in vivo*. In the *ex vivo* approach, target cells are removed from patients, transduced with the desired gene and replaced in the subject. In this respect, the therapeutic entity comprises the engineered cells. Although *ex vivo* gene transfer offers more efficient gene transduction and easier propagation for generating higher cell doses, it has the obvious disadvantages of being patient specific and more expensive due to manufacturing and quality control requirements. Conversely, the *in vivo* approach involves direct administration of the gene transfer vehicles to patients and is therefore not patient specific and potentially less costly.

Clinical studies currently conducted on a wide range of diseases are listed in Table 1. Approximately two-thirds of the approved protocols are directed at cancer therapy. The remaining applications mostly concern the treatment of inherited monogenic diseases (eg cystic fibrosis and haemophilia) and infectious diseases (eg AIDS/HIV infection). This distribution may reflect the lack of effective alternative therapies, thereby increasing the likelihood of

regulatory approval. Early clinical trials frequently involved cells engineered *ex vivo* with retroviruses expressing reporter genes. These studies assessed the pharmacokinetics and biodistribution of the engineered cells in patients with cancer or inherited monogenic disorders. However, most of the recently approved protocols have therapeutic intent and most protocols focus on the destruction of diseased cells, as opposed to long-term restoration of defective or missing genes. This trend has understandably resulted from limited choices of gene delivery vectors and the lack of sustained expression systems. Table 2 summarises the current clinical trials conducted in major disease categories.

Methods of gene transfer

The major approaches to gene delivery fall into two categories: viral and non-viral. Non-viral approaches usually include: 1) direct transfer of naked DNA molecules; 2) transfer of DNA complexed with cationic lipids; 3) transfer of particles comprising DNA condensed with cationic polymers or liposomes (Wolff and Trubetskoy 1998); and 4) physical methods involving needle-free injectors and electroporation. Non-viral approaches yield less efficient transfection than viral approaches and yield mostly transient expression. However, they have no insert-size limitation, are less immunogenic and are easier to manufacture than viral vectors. Conversely, many different viruses have been adapted as gene transfer vectors. The most commonly used vectors are adenoviruses, retroviruses (including the recently

Table 2 Current clinical trials in major disease categories

<i>Non-cancer related</i>	<i>Cancer-related</i>
Acute hepatic failure	Bladder 2
Anaemia	Breast 17
Bone disorders	Cervical 2
Cardiovascular	Central nervous system 36
Infectious Disease	Colon 13
Inherited autosomal dominant	Leukaemia 34
Inherited autosomal recessive	Liver 23
Mesothelioma	Lung 23
Multiple sclerosis	Ovarian 24
Myopathy	Prostate 40
Neurologic disorder	Renal cell 6
Rheumatoid arthritis	Sarcoma 1
X-linked diseases	Skin 63
Other	Solid Tumours 5
	Squamous cell head neck 71
Total	166^a
	Total
	360^a

Source: <http://www4.od.nih.gov/oba>^a Some trials involving multiple diseases

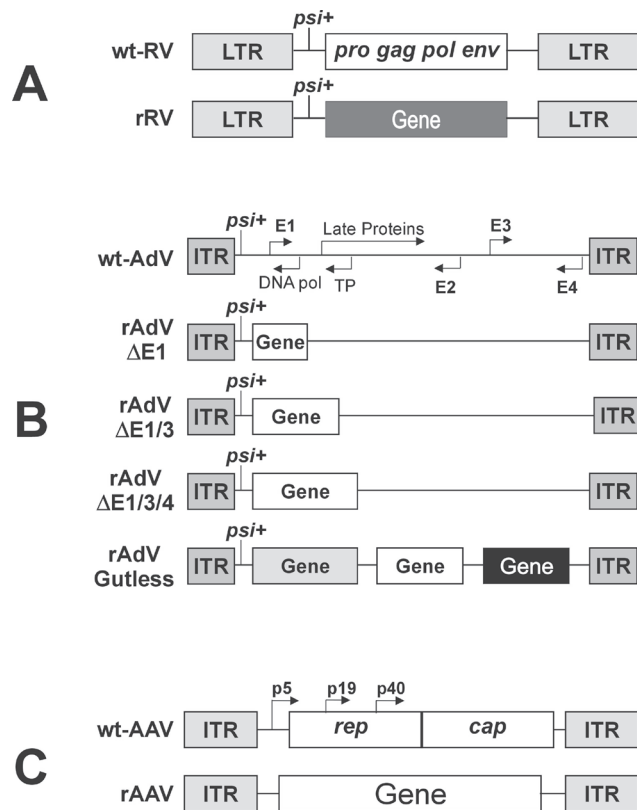


Figure 1 Schematic genomic structures of commonly used viral vectors. (A) Retroviral vector: wild-type (wt) retroviral vector contains genes *gag*, *pol* and *env* that are essential for propagation. In the recombinant retrovirus (rRV), a transgene is substituted for these elements, which are subsequently provided in trans from packaging cells. Lentiviral vectors are structurally similar. (B) Adenoviral vector: wt expresses both early and late genes. The various recombinant adenoviral vectors (rAdV) substitute a transgene in place of *E1*, *E3*, and/or *E4* genes. The rAdV DE1/3/4 and rAdV gutless vectors require packaging cell lines or a helper virus to supply missing elements in trans. (C) Adeno-associated viral vector: wt contains *rep* and *cap* that encode replication and structural proteins, respectively. Recombinant adeno-associated viral vector (rAAV) is generated with a helper adenovirus and an expression vector that supplies deleted proteins in trans. LTR: long terminal repeats; ITR: inverted terminal repeats; *psi*: packaging signal.

developed lentiviral vectors) and adeno-associated viruses (Robbins et al 1998)(Figure 1). Substantial effort has also been devoted to the development of poxviruses, or vaccinia viruses and the herpes simplex virus (Sanda et al 1999; Krisky et al 1998). As summarised in Table 3, the leading viral vectors possess the highest efficiency of gene transduction; whereas, their main disadvantages stem from their limited capacity to accommodate large inserts, their immunogenicity and the difficulty in manufacturing high quality stocks. Despite these shortcomings, viral vectors remain one of the most viable gene delivery approaches for gene therapy. In this review, we focus primarily on the development and application of various viral vectors for gene transfer, with an emphasis on the most commonly used ones: retroviral vectors (including lentiviral vectors), adenoviral vectors, adeno-associated viral vectors and herpes simplex viral vectors.

Retroviral vectors

Biology of retroviruses

Retroviral vectors are used as effective vehicles to deliver and integrate genes into host cells. They remain a popular means of gene transfer, as is evident from the high percentage of retroviral vectors used in clinical trials. Retroviruses are single-stranded linear RNA molecules complexed as homodimers within a nucleocapsid envelope. Viral envelope glycoproteins mediate entry into host cells, and viral reverse transcriptase converts the RNA genome into double-stranded DNA that integrates permanently into the host genome (Weiss 1998). Virions approximately measure 120nm in diameter and contain a genome of 7 – 11kb. Although several types of retroviruses have been utilised for gene therapy, the murine leukaemia virus (MuLV) has been the one most widely used for gene therapy trials. Based on its host tropism, MuLV has been classified into three subgroups: xenotropic, able to infect cells from many mammals except rodents; ecotropic, only infects rodents; and amphotropic, infects both rodents and non-rodents alike. Amphotropic MuLV enjoys widespread applications in clinical trials due to its diverse tropism.

All retroviral genomes have a similar structural organisation (Figure 1A). Each contains four genes essential to propagation: *gag*, *pro*, *pol* and *env*. The *gag* gene encodes structural proteins including the matrix proteins that surround the viral core and fortify the viral particle. The *pro* gene serves as a template for a protease that cleaves *gag* and *gag-pol* products. It plays a vital role in viral particle maturation, both pre- and post-budding. The *pol* gene encodes a reverse transcriptase that converts RNA to proviral DNA, as well as integrase, which promotes proviral DNA integration into the host genome. The *env* gene encodes components of the glycoprotein envelope, a viral shell that mediates translocation of the virus to the host cell. Flanking these basic genomic components are two long terminal repeats (LTRs), cis-acting sequences that control viral gene expression, reverse transcription and integration into host chromatin (Coffin 1996).

The unique life cycle of retroviruses make them well suited for gene delivery. Infection begins with entrance into the host cell as mediated by viral glycoprotein interactions with target cell receptors. Virus-receptor interactions trigger fusion of viral and host cell membranes and the subsequent absorption of the viral particle, resulting in release of the viral core into the cytoplasm. Subsequently, a pre-integration complex containing the viral genome is formed after partial degradation of the viral core and translocation to the nucleus.

Table 3 Comparison of commonly used viral vectors for gene transfer

<i>Viral vectors</i>	<i>Adenoviral</i>	<i>AAV</i>	<i>Retroviral</i>	<i>Lentiviral</i>	<i>HSV</i>
Viral genome	DNA	DNA	RNA	RNA	DNA
Host spectrum	broad	broad	restricted ^b	broad ^b	restricted
Efficiency of gene transfer	very high	high/moderate	low	low	moderate
Integration to host genome	no	yes/no ^a	yes	yes	no
Duration of expression	weeks–months	long-term	long-term	long-term	days–weeks
Construction procedure	easy	established	established	difficult	difficult
Transgene size	5–36kb ^c	4–5kb	4–5kb	8–9kb	up to 50kb
Vector titre (pfu/ml)	high (>10 ¹¹)	low (<10 ⁹)	low (<10 ⁷)	low (<10 ⁶)	high (10 ¹⁰)
Cell proliferation required	no	no	yes	no	no
Immunogenicity	high/low ^c	low/rare	rare	rare	high
Frequency of use in trials	high	moderate	high	none/low	low

^a Most current rAAV vectors do not integrate into the host chromosome.

^b VSV-G pseudotyped vectors exhibited broader host range.

^c Gutless vectors exhibit large cloning capacity and reduced immunogenicity.

Nuclear entry differs among retroviruses. In dividing cells MuLV translocates to the nucleus only after nuclear membrane disruption during mitosis because it is unable to cross nuclear pores on its own. After entry into the nucleus, retroviral RNA is used to generate double-stranded proviral DNA that then integrates into the host genome. The host cells then produce the nascent viral genome RNA and all of the viral proteins needed to assemble new virions. After splicing occurs in the nucleus, the viral materials are transported into the cytoplasm where they bud from the cell membrane and produce new viral particles.

Development of recombinant retroviral vectors

To serve as safe and effective gene delivery vehicles, retroviruses must be modified to reduce their pathogenicity, as well as their ability to replicate and infect surrounding cells. Thus, recombinant retroviral vectors are usually engineered in such a way that the structural genes *gag*, *pol*, and *env* are deleted from a retroviral genome and substituted with a therapeutic gene. To generate recombinant viruses, the structural genes are supplied in trans in helper cells or packaging cells, in which the structural genes cannot be packaged into retroviral particles because they lack the *psi*-packaging signal (Markowitz et al 1988). However, earlier generations of packaging cells were shown to produce replication-competent retroviruses (RCR) after a single recombination event between homologous sequences in the constructs providing packaging function and those expressing the therapeutic genes. This results in restoration of the *psi*-packaging signal for the structural genes. To address the risk of RCR production, newer generations of packaging cell lines provide viral genes through expression of heterologous

transcription signals from two separate constructs lacking the viral cis-acting sequences (Chong et al 1998; Chong and Vile 1996; Danos and Mulligan 1988). Although the biosafety of the retroviral vectors has been greatly improved, RCR viruses generated in these packaging cell lines have been detected (Chong et al 1998). The use of a heterologous promoter, such as the cytomegalovirus (CMV) promoter, can result in robust expression of packaging elements, while reducing the chance of recombination events (Rigg et al 1996; Soneoka et al 1995). The substitution of different viral species LTRs to provide packaging functions may also improve the biosafety of retroviral generation (Cosset et al 1995).

In addition to improving the biosafety of packaging lines, producer cells capable of generating high viral titres must be established. Currently, viral titres can reach concentrations of 10⁷–10⁸ pfu/ml. While sufficient for the in vitro transduction of cells and certain ex vivo applications, such titre levels are not sufficient for in vivo studies. Significant improvements in viral titres have been accomplished by using selectable markers to enrich effective viral producer cells in vitro (Cosset et al 1995). Viral production can also be improved by using packaging cells that do not secrete molecules, known to inhibit infectivity, such as proteoglycans (Le Doux et al 1996). Another factor limiting the in vivo infective potential of retroviruses is serum complement activation (Rother et al 1995; Takeuchi et al 1996; Welsh et al 1975). Human cell lines lacking *alpha-gal* expression, a ubiquitous foreign epitope that activates the complement cascade, have been exploited to create complement-resistant packaging lines (Pensiero et al 1996; Rollins et al 1996). In addition, the use of endogenous cellular factors that curb complement activation, such as decay accelerating factor (DAF) are being used to down-regulate complement-

mediated lysis of viral particles (Hiasa et al 1999; Spitzer et al 1999).

The need for an expanded viral host range and more targeted infectivity has led to pseudotyping, a technique whereby one viral *env* is substituted for a heterologous one. Chimeric *env* sequences have been engineered that contain sequences derived from CD4, single-chain antibodies, or the polypeptide erythropoietin (Chu and Dornburg 1997; Han et al 1995; Kasahara et al 1994; Russell et al 1993; Valsesia-Wittmann et al 1994; Young et al 1990). When expressed and packaged in the lipid envelope of viral particles, chimeric *env* proteins bind alternative cell receptors and redirect viral tropism. Unfortunately, while viral infection with pseudotyped *env* occurs, it is relatively inefficient. In order to achieve efficient infection, wild-type *env* possessing particles must also be present, and even then, only low viral titres have been obtained. Using pseudotyping of the retroviral core with the G-protein of vesicular stomatitis virus (VSV-G), viral titres may exceed 10^{10} pfu/ml with increased efficiency of infection and a stabilised viral envelope. Furthermore, the tropism of VSV-G pseudotyped viruses is broader, including primary hepatocytes, fibroblasts, CD34⁺ and CD38⁺ cells (Burns et al 1993).

Applications of retroviral vectors

Retroviral vectors have been widely used in clinical trials. Currently, 168 clinical protocols employ retroviral vectors for gene therapy. The initial clinical studies on retroviruses sought to determine the biosafety of retroviral gene therapy and kinetics of transduced cells in human subjects. One such investigation used tumour-infiltrating lymphocytes (TIL) from patients with advanced melanoma and transduced them *ex vivo* with a vector expressing the neomycin resistance gene (Rosenberg et al 1990). The first therapeutic clinical trial attempted to treat severe combined immunodeficiency (SCID). SCID is caused by an adenosine deaminase deficiency (ADA) that reduces the viability of T cells, leaving patients immunodeficient. When T cells from these patients were transduced *ex vivo* with the *ADA* gene, their function and immune response was restored (Anderson et al 1990; Rosenberg et al 1990); however, repeat *ex vivo* treatments were needed to sustain this response. With the success of the first gene therapy trial, attempts were initiated to treat other monogenic diseases with *ex vivo* retroviral gene therapy including Gaucher's disease, Hurler syndrome, Fanconi anaemia, haemophilia, and hypercholesterolaemia (Fairbairn et al 1997; Fu et al 1997; Grossman et al 1995; Lu et al 1993).

The intrinsic preference of retroviruses for dividing cells has made retroviral vectors particularly attractive for treatment of human cancers. Numerous attempts have been made to boost or amplify host anti-tumour immune defenses by creating autologous tumour cell vaccines by *ex vivo* transduction of TILs or tumour cells with retroviral vectors expressing immunomodulators, such as IL-2 and tumour necrosis factor- α (TNF- α) (Cowan et al 1999; Nemunaitis et al 1998; Palmer et al 1999; Tan et al 1996). Such approaches have been used in the treatment of melanoma, renal cell carcinoma and neuroblastoma (Gansbacher et al 1992). Other cytokine immunomodulators, including GM-CSF, Interferon and IL-7, have been used in retroviral gene therapy cancer studies as well. Autologous bone marrow cells transduced with multi-drug resistant genes via *ex vivo* retroviral gene transfer have been rendered chemoresistant (Cowan et al 1999; Moscow et al 1999). Another anti-cancer strategy is to restore endogenous tumour suppressor genes in neoplastic cells. For instance, the reintroduction of wild-type *p53* into non-small cell lung cancer cells has been conducted (Roth et al 1996). More recently, the use of *in vivo* retroviral gene therapy has been used to treat brain tumours. This represents a particularly attractive approach given that the neoplasm rests in a field of non-dividing cells (Oldfield et al 1993; Palu et al 1999; Ram et al 1997). Producer cells or purified vectors containing the cytotoxic gene *HSV-tk* or the immunomodulator *IL-2*, have been stereotactically injected into a brain mass. It remains too early to comment on the overall efficacy of these treatments.

Advantages and limitations of retroviral vectors

The popular choice of retroviral vectors for use in gene therapy can be attributed in part to the extensive research on retrovirus biology that has resulted in genome characterisation and life cycle elucidation. These investigations have revealed a number of advantages of retroviral vectors. First, the recombinant retroviral genome usually integrates into the host genome, such that transgene expression can be sustained over long periods. Second, they can accommodate foreign genes up to 7–8kb in size. Third, recombinant viral vectors are relatively easy to manipulate and engineer. Fourth, they can infect a broad range of cell types with high efficiency.

A number of shortcomings, however, have limited the widespread use of retroviral vectors in gene therapy trials. First, the broad cell tropism of retroviral vectors reduces their tissue specificity, although this is not unique to retroviruses. Second, the inability of retroviral vectors to infect non-

dividing cells hampers their use in tissues where cells are mostly quiescent. As discussed below, the recent development of lentiviral vectors may circumvent this shortcoming. Third, the potential for random integration into the host genome and disruption of host gene expression may be detrimental to host cells, although such an effect has not been observed or reported thus far. The Biological Response Modifiers Advisory Committee, a group advising the United States Food and Drug Administration on gene therapy, has been charged with determining the long-term (ie 15-year follow-up) safety risks of gene therapy to patients (Birmingham 2001). Fourth, the production of wild-type virus (ie RCR) is always a possibility during recombinant virus amplification. Fifth, it has been reported that the expression of transgenes decreases over time. Although the mechanism for this is not clearly understood, it has been postulated that methylation of the 5'LTR in host cells may be partially responsible for diminished transgene expression (Lengauer et al 1997). Finally, it remains a challenge to produce high titre virus stocks (as compared with adenoviral vectors, see Section 3) although significant improvements have been made when VSV-G pseudotyping vectors are used.

Development and applications of lentiviral vectors

Lentiviruses are a subtype of retroviruses that includes human immunodeficiency viruses type I (HIV-1) and type 2 (HIV-2). They are currently being developed as gene transfer vehicles. Worldwide interest in HIV research has facilitated our understanding of this viral class for use in gene therapy. As with other retroviruses, the lentiviral genome contains the genes *gag*, *pro*, *pol* and *env*. In addition, lentiviruses contain accessory genes that yield a more complex genome and replication life cycle (Coffin 1996). This added complexity enables the lentiviruses to infect non-dividing cells. Thus, terminally differentiated cells or those that have low mitotic indices (such as hepatocytes, neurons, myocytes and haematopoietic stem cells) are appealing targets for these vectors. While the precise mechanism by which lentiviruses traverse nuclear pores remains unclear, it has been speculated that some of the viral proteins may play an important role in this process. The Vpr protein is known to bind directly to nuclear pores (Vodicka et al 1998), while the integrase and Gag proteins drive nuclear translocation (Bukrinsky et al 1993; Gallay et al 1995; Heinzinger et al 1994; Kondo et al 1995). It has been demonstrated that each of these proteins can successfully drive localisation independent of each other; however, further investigation is required to determine how

these factors work and what controls viral transportation. In addition to the control of nuclear localisation signalling, *tat* and *rev* gene products play a crucial role in the lentivirus life cycle. The *tat* protein activates a promoter in the 5' HIV LTR that initiates efficient viral RNA production. The *rev* protein binds the *rev*-responsive element (*rre*) post the transcription of viral RNA and catalyses viral transport out of the nucleus (Cowley 2001).

Lentiviral vectors represent an appealing modality of gene transfer because they are able to infect dividing and non-dividing cells alike, and can accommodate larger transgenes (up to 10kb) than their oncoretrovirus counterparts (Poeschla et al 1996). Many of the same problems hindering retroviral vectors apply to lentiviruses as well, such as the potential for insertional mutagenesis caused by random genomic integration, the inability to target specific cell types *in vivo* and the difficulty generating high titres of virus for *in vivo* experiments. In addition, the generation of stable packaging cell lines has been problematic, likely due to the toxicity of some HIV proteins. More importantly, the potential generation of an RCR HIV is especially worrisome, as it is a deadly human pathogen without effective anti-viral drugs. While no RCRs have been detected among lentiviral vectors generated to date, careful surveillance of therapeutic stocks using a PCR-based detection method for potential wild-type recombinant viruses is essential to safeguard the biosafety of these vectors (Kafri 2001).

The limited understanding of the viral factors required for packaging of lentiviruses made initial attempts to generate packaging cell lines difficult. Initial HIV vectors were constructed using a heterologous viral promoter from simian virus 40 spliced into the *env* gene (Helseth et al 1990; Page et al 1990). These *env* protein-deficient vectors were supplemented *in trans* by helper cells. This construct had limited potential owing to its narrow cell tropism (CD4+ cells only) and low viral titres. Pseudotyping with MuLV envelope glycoprotein (Page et al 1990) and VSV-G (Yee et al 1994) produced vectors with more diverse tropisms and, in the latter case, yielded a vehicle with a higher titre and greater stability. Newer generations of HIV vectors mimic oncoretroviral vectors in which most viral genes are deleted, leaving only essential *cis*-acting elements. HIV vectors have been utilised to efficiently infect hepatic, retinal and muscle cells (Kafri et al 1997; Miyoshi et al 1997). Studies of animal models have shown that HIV-1 vectors were not only capable of infecting quiescent neural cells, but expressed transgenes for months (Blomer et al 1997; Naldini et al 1996a; Naldini et al 1996b). In fact, stable long-term transgene expression of

neural cells has been reported in animal models of Parkinson's disease (PD) (Kordower et al 2000), retinal photoreceptor degeneration (Takahashi et al 1999), type VII mucopolysaccharidosis (Bosch et al 2000) and metachromatic leukodystrophy (Consiglio et al 2001).

Adenoviral vectors

Biology of adenoviruses

Adenoviruses are non-enveloped, linear, double-stranded DNA viruses roughly 36kb long (Figure 1B). Adenoviral genes are defined temporally in relation to viral DNA replication (eg early, delayed early, or late). Early gene products drive gene transcription, viral DNA replication, inhibition of host cell apoptosis and host immune suppression; whereas late genes encode proteins vital for viral packaging and virion formation (Flint and Shenk 1997). One such early gene *E1A*, is a vital transcriptional activator that encodes factors that induce a G1-S transition in host cells (Flint and Shenk 1997). The life cycle of the adenovirus is dependent on its penetration into host cells. To achieve entry, the adenoviral outer fibre protein knob binds tightly to cell surface receptors, such as MHC-I α 2-domain or CAR (coxsackievirus and adenovirus receptor) (Bergelson et al 1997; Hong et al 1997). This initial coupling is followed by additional interactions with surface proteins triggering receptor-mediated endocytosis (Huang et al 1995; Walters et al 1999). The virus then translocates to the nucleus where viral genes are replicated and/or transcribed using host machinery. The assembly of viral progeny induces host cell death and release of the virion.

Technological advances in generating recombinant adenoviral vectors

Two main methods of recombinant adenovirus production have been developed. The first employs direct ligation of adenoviral DNA fragments, except for the E1-region, to restriction endonuclease cleaved fragments with pre-inserted transgenes (Ballay et al 1985; Mizuguchi and Kay 1999; Rosenfeld et al 1991). Multiple problems have made this approach technically challenging, including: 1) the scarcity of unique restriction sites, 2) the challenge of purifying large adenoviral genomic DNA fragments, and 3) the low efficiency of ligating large fragments. A second method, that is used more widely, utilises homologous recombination in mammalian packaging cells to complement replication defective adenoviruses. For this approach a two-vector system, with 'shuttle' and 'helper' plasmids, is required

(McGrory et al 1988). The shuttle vector contains a transgene whose expression is driven by its own regulatory elements. The helper vector supplies the adenoviral genomic backbone. The helper is not capable of producing viruses, because it contains either the whole but un-packagable viral genome or most of the viral genomic backbone with deletion of the *E1* and other essential genes. The introduction of both vectors into the packaging cells leads to the generation of recombinant adenoviruses through homologous recombination.

The recombinant adenovirus contains a transgene that replaces the adenoviral early genes (eg *E1A* and *E1B* genes). Although this method has been successful in generating adenoviruses, it remains time-consuming and laborious due to the inefficiency of homologous recombination in mammalian cells. To improve recombination efficiency between transgene-containing shuttle vectors and adenoviral backbone vectors, Ketner and colleagues were the first to exploit the highly efficient homologous recombination machinery in *Saccharomyces cerevisiae* to produce yeast artificial chromosome (YAC) clones containing human adenoviral genome (Ketner et al 1994). Since then, there have been substantial efforts in developing more efficient and simplified systems to generate recombinant adenoviruses (Chartier et al 1996; He et al 1998). Currently, one of the most widely used techniques generates recombinant adenovirus vectors in certain strains of *E. coli* cells, which are proficient in generating stable recombinants under proper selective growth conditions (He et al 1998). Alternatively, efficient recombination mediated by the Cre recombinase/loxP site-specific recombination system also has been reported (Hardy et al 1997; Ng et al 1999). By improving recombination efficiency, the technological advances of the past few years have circumvented the rate-limiting step in recombinant adenoviruses production. These steps make it possible to generate a large quantity of adenoviral vectors in an efficient and predictable fashion.

Significant progress has been made in reducing the cytotoxic effects of adenoviral vectors, while reducing the host immune response and accommodating larger transgenes. For example, double deletion or triple deletion adenoviral vectors can package up to 10kb of foreign DNA and have been shown to lengthen transgene expression and diminish cytotoxic effects in vivo (Bett et al 1995; Engelhardt et al 1994a; Engelhardt et al 1994b; Gao et al 1996; He et al 1998; Schowalter et al 1999; Yang et al 1994). Recently, the gutless adenoviral vector has emerged as one of the most promising vectors, possessing a carrying capacity of up to 35kb of foreign DNA (Fisher et al 1996; Kochanek et al 1996; Parks

et al 1996). In this system, a helper virus provides essential functional and structural genes in trans, thus most of the viral genome is removed to provide room for exogenous DNA. The gutless vector system was characterised by reduced immunogenicity and cytotoxicity in vivo, yet provided long-term expression of multiple transgenes (Morral et al 1998; Morsy et al 1998). Currently, the main drawback of the gutless system is the difficulty in completely removing helper virus from recombinant virus stocks.

Applications of adenoviral vectors

The majority of adenoviral cancer gene therapy has focused on the in vivo delivery of immunomodulators (such as IL-2, IL-12, FasL), tumour suppressor genes (such as *p53*), antiangiogenic genes (such as *VEGF*) and drug-sensitive genes (such as the herpes virus *thymidine kinase (TK)*) to tumour tissue (Herman et al 1999; Molnar-Kimber et al 1998; Morelli et al 1999; Motoi et al 2000; Roth and Cristiano 1997; Rubinchik et al 2000; Serman et al 1998b). A diverse assortment of neoplasms have been treated with adenoviral based gene transfer, such as melanoma, prostate cancer, mesothelioma, pancreatic cancer, metastatic liver cancer secondary to colorectal cancer, lung cancer, neuroblastoma, glioblastoma, ovarian cancer, and squamous cell carcinoma of the head and neck (Habib et al 1999; Herman et al 1999; Molnar-Kimber et al 1998; Schuler et al 1998; Serman et al 1998a; Serman et al 1998b).

Much progress has been made towards engineering conditionally replicative adenoviruses (CRAd) for cancer gene therapy. This method produces viruses that only replicate in tumour cells and exploit wild-type adenoviral cytotoxicity to induce neoplasm death and deliver a therapeutic gene (for review see Douglas and Curiel 1997). For example, a CRAd dependent on *p53* tumour suppressor gene status has been constructed. The *E1B-55kDa*-deleted adenovirus replicates selectively in *p53*-deficient cells (Bischoff et al 1996). The wild-type *E1B55k* gene product binds *p53* and inactivates it, driving viral replication. Thus, only cells devoid of *p53* (ie cancer cells) will allow vector replication. However, in recent studies of an *E1B-55kDa*-deleted adenovirus, the restriction of viral replication to *p53*-deficient cells occurred only in certain cell lines. CRAds used to treat breast, hepatocellular and prostate cancer exploit viruses with promoters that respond only to tissue specific factors (Hallenbeck et al 1999; Rodriguez et al 1997; Yu et al 1999). For example, in a CRAd used to treat breast cancer, the expression of adenoviral *E1A* and *E4* genes are under the control of a promoter containing estrogen-responsive

elements (Hernandez-Alcoceba et al 2000). This promoter induced transcriptional activation of the *E1A* and *E4* genes only in cells that expressed the oestrogen receptors (OR), thereby targeting OR⁺ breast cancer cells. A similar cell-type specific approach has been developed for brain tumours, using a CRAd that was also inducible (Smith-Arica et al 2000). Finally, a CRAd with double suicide genes was developed that had significant anti-tumour activity and significantly potentiated the efficacy of radiation therapy. This CRAd expressed a cytosine deaminase/herpes TK fusion protein. The cytosine deaminase and herpes TK convert systemically delivered, innocuous prodrugs 5-fluorocytosine and ganciclovir, respectively, into toxic metabolites (Rogulski et al 2000). Adenoviral vectors have also been widely used in gene transfer studies directed at several monogenic diseases, including cystic fibrosis (Zuckerman et al 1999), muscular dystrophy (Douglas and Curiel 1997) and haemophilia (Zhang et al 1999). With the discovery of the cystic fibrosis transmembrane conductance regulator (CFTR), the gene whose dysfunction results in the development of cystic fibrosis, considerable excitement was generated regarding the potential for gene therapy in the treatment of the condition. Adenoviral vectors expressing CFTR were developed and tested in phase I clinical studies to assess their biosafety and ability to generate the desired gene product. Despite some promising initial results, the vectors failed to generate sustainable gene expression and elicited a significant immune response. Thus, additional administrations may not lead to repetitive expression because of host immune responses (Harvey et al 1999).

Recently, the efficacy of adenoviral vectors has been demonstrated in several models of neurodegenerative diseases including Huntington's disease (HD) and PD (Barkats et al 1998; Smith, 1998). HD is an autosomal dominant genetic disorder leading to the degeneration of striatal GABA-nergic output neurons. The adenoviral delivery of brain-derived neurotrophic factor (BDNF) to rat models improved disease symptomatology (Bemelmans et al 1999). In a rat model of PD, adenoviral vectors expressing either tyrosine hydroxylase, superoxide dismutase or glial-derived neurotrophic factor improved the survival and function of dopaminergic cells (Corti et al 1999). Adenoviral vectors have also been shown in animal models to deliver therapeutic genes effectively for several cardiovascular diseases, such as atherosclerosis, cerebral ischaemia, familial hypercholesterola emia, hypertension and cardiac arrhythmia (Gerard and Collen 1997; Kevin Donahue et al 2000; O'Brien and Simari 2000; Papadopoulos et al 2000; Stein et al 2000;

Tangirala et al 1999). Lastly, adenovirus-mediated gene transduction has been explored for a variety of genetic and metabolic liver disorders, such as lysosomal storage diseases, glycogen storage diseases, phenylketonuria and Tay-Sachs disease (Amalfitano et al 1999; Eto and Ohashi 2000; Guidotti et al 1999; Nagasaki et al 1999; Stein et al 1999; Ziegler et al 1999; Zingone et al 2000).

Advantages and limitations of adenoviral vectors

A number of attributes make adenovirus vectors well suited for gene therapy (Breyer et al 2001). Infection with adenoviral vectors reliably results in high levels of gene expression and high viral titres can easily be obtained. In addition, with the recent advent of 'gutless' adenoviral vectors up to 35kb of foreign genetic material can be accommodated. While recombinant adenoviral vectors have become increasingly popular gene delivery vehicles, there are a number of limitations. First, adenoviral vectors tend to elicit strong immune responses in vivo; second, the vectors are non-specific with a wide tropism; third, adenoviral vectors are unable to maintain long-term gene expression. It is already known that 55% of the general population have had prior exposure to and, therefore, have already generated antibodies against adenoviruses (Chirmule et al 1999). However, it remains to be seen if these antibodies will significantly hinder systemic administration of adenoviral gene therapy. In the case of tumour treatment, this issue can be circumvented by direct tissue injection. With regard to systemic therapy, several efforts have been made to reduce the immunogenicity of adenoviral vectors by: 1) controlling host immune responses at the time of infection, 2) deleting viral genes encoding immunogenic proteins, and 3) altering capsid components to circumvent pre-existing humoral immunity.

The host immune response at the time of infection is the most vigorous and represents the best window of opportunity to diminish adverse immunogenic responses. To accomplish this, the transient blockade of cell adhesion and co-stimulatory molecules, such as the CD40 ligand, has been investigated (Yang et al 1996). The use of immunomodulating cytokines, such as IL-10 and IL-12, have also been used to upset balanced T-helper (Th) activation towards either the cytotoxic T-helper cell 1 (Th1) or the humoral T-helper cell 2 (Th2) subset, thereby reducing either antibody production or cellular immune response, respectively (Qin et al 1997; Yang et al 1995). Another immunomodulating cytokine, TNF- α , plays a prominent role in the adenovirus-induced immune response, and inhibition of the TNF- α pathway, by,

for example, injection of soluble TNF receptor (Zhang et al 1998) may potentially diminish host cell immune responses. Over-expression of E3B-coded antagonists (Ilan et al 1997) has also been used as an alternative therapy.

Immunogenicity elicited by adenoviral vectors can be circumvented by changing vector design. For instance, a new generation of adenoviral vectors has been constructed by deleting the *E1*, *E2* and *E4* genes in order to avoid expression of immunogenic viral proteins in host cells (Ferry and Heard 1998). Alternatively, constitutive expression of E3 gp19K protein in *E1*-deleted vectors has provided encouraging results with more stable transgene expression in the liver and lung of animal models (Bruder et al 1997; Ilan et al 1997). gp19K inhibits the transport of major histocompatibility complex class I molecules to the cell surface, leading to the impairment of the function of antigen-presenting cells and the reduced clearance of adenoviral infected cells by cytotoxic lymphocyte (CTL)-mediated immune responses (Beier et al 1994; Lee et al 1995). The recently developed gutless adenoviral vectors that have most or all adenoviral genes deleted, have significantly reduced immunogenicity, yet are capable of prolonged expression of homologous transgenes in mice (Chen et al 1997; Morsy et al 1998; Schreiber et al 1999). Interestingly, a conceptually different approach was employed to excise significant portions of viral genes from the vector backbone, in which the complete E2 region was flanked with loxP sites and removed with Cre recombinase (Lieber et al 1996). The resulting gutless virus minichromosome can be further stabilised with E2B-encoded preterminal protein (Lieber et al 1997; Lieber et al 1996). Although low levels of contaminating helper virus were present, gutless vectors did not elicit a strong cellular immune response. In contrast, they do induce a humoral response against the viral capsid components at the time of injection.

Alteration of viral capsid components may provide another alternative to limit the humoral response to the commonly used adenovirus serotype 5. Chimeric vector capsids produced by combining different adenoviral subtype hexon monomers were shown to overcome neutralising antibodies in C57BL/7 mice primed with Ad5 (Roy et al 1998). Furthermore, the insertion of an Arg-Gly-Asp (RGD) motif into the fibre gene of an adenoviral vector has generated a vector with expanded cell tropism and enhanced transduction efficiency in primary tumour cells. Of note, chemical cross-linking between polyethylene glycol (PEG) and adenoviral vectors has also been shown to protect viral particles effectively from antibodies both in vitro and in vivo (O'Riordan et al 1999).

Adenoviral vectors transfer genes into a diversity of cell types. This broad tropism also represents a drawback when systemic gene delivery to a specific tissue is desired. Several strategies have been developed to address this issue. Earlier attempts to control adenovirus tropism usually relied on the use of bispecific binding molecules that block native receptor binding, thereby redirecting virus binding to a tissue-specific receptor. For example, the F_{ab} fragment of a neutralising monoclonal antibody against the fibre protein conjugated to folate was shown to redirect adenoviral infection to cells expressing folate receptors (Douglas et al 1996). Similarly, a bispecific antibody against both the fibre protein and the epidermal growth factor receptor (EGFR) was able to abrogate native adenovirus binding and redirect viral infection to human glioma cells via EGFR (Miller et al 1998). Alternatively, redirected adenoviral tropism can also be achieved by modifying the fibre protein. It has been reported that viral vectors containing chimeric fibre coat proteins with peptide ligands allowed specific binding to heparan sulphate and integrin receptors (Wickham et al 1997). More recently, a novel strategy for cell type-specific gene delivery has been developed by modifying adenoviral vectors with biologically selected peptides (O’Riordan et al 1999; Romanczuk et al 1999). Novel peptide ligands that specifically bind to airway epithelial cells were first isolated by biopanning the cells against a phage display library. The peptide with the most effective binding was then coupled to the surface of an adenoviral vector using bifunctional PEG molecules.

Long-term expression of transgenes is desirable for replacement gene therapy. Several strategies have been developed to address the drawback of adenovirus-mediated transient gene expression. For example, a chimeric adenoviral-retroviral vector has been constructed in order to maintain transgenes within actively dividing cells (Bilbao et al 1997; Duisit et al 1999; Feng et al 1997). This chimeric virus was shown to infect cells and produce recombinant retroviruses that can then infect surrounding cells and integrate into host chromosomes. Similarly, a hybrid adenoviral/adeno-associated virus (AAV) was engineered and has been shown to result in the incorporation of the transgene at a specific locus of human chromosome 19 (Lieber et al 1999). The major difference between the two types of chimeric viruses is that the AdV/AAV vector may also maintain efficient and lasting transgene expression in nondividing cells. Others have also explored the possibility of long-term expression mediated by the EBNA1/OriP episomal replication system derived from Epstein-Barr virus (Benihoud et al 1999).

Adeno-associated viral vectors

Initially discovered as a ‘defective satellite virus’ contaminating laboratory stocks of adenovirus (hence its name), AAV were later classified as members of the Parvovirus family (Monahan and Samulski, 2000). AAV are single-stranded DNA dependoviruses. Each viral particle contains either a positive or negative sense strand. There are six known viral serotypes with type AAV-2 being the most commonly studied and used. As shown in figure 1C, the AAV genome is composed of two genes, *rep* and *cap* that encode replication and structural proteins, respectively (Muzyczka, 1992; Russell and Kay 1999; Tal 2000). The genome is flanked by viral inverted terminal repeats (ITRs). An internal promoter and transgene replace the viral genomic genes to produce a recombinant adeno-associated virus (rAAV). AAV requires either adenoviral or herpes viral elements to produce a productive infection (Muzyczka 1992). In particular, adenoviral E1B and E4 are required, as E4 controls the synthesis of the second complementary AAV DNA strand (Fisher et al 1996). After a double-stranded DNA molecule is created by self-dimerisation or E4-driven synthesis of a complementary strand, AAV becomes transcriptionally competent, leading to viral replication and assembly of viral particles with a helper virus.

AAV vectors are attractive as gene delivery systems for a number of reasons. They can infect dividing and non-dividing cells with similar efficiency and have a broad tropism. AAVs are integrating vectors with a potential for persistent transgene expression. Furthermore, AAVs are not associated with any known human diseases and are only mildly immunogenic (Blacklow et al 1968). Potential may exist for targeted integration of AAVs into the host genome, thus eliminating the chance of insertional mutagenesis. Under the direction of *rep* gene products, wild-type AAV integrates into 19q chromosomal loci with 70% likelihood (Kotin et al 1990). However, current rAAV vectors lack directed integration because *rep* genes are excised. One of the significant shortcomings for AAV vectors is that their accommodation capacity is small (4.1–4.9kb), although the use of heterodimer vectors could effectively double its capacity (Nakai et al 2000; Sun et al 2000; Yan et al 2000). Another problem involving rAAV systems is the difficulty in producing high titre virus stocks. This has improved somewhat with the development of helper plasmids to replace adenoviruses. Using 293 packaging cells co-transfected with rAAV and helper plasmids, it has been possible to generate higher titre stocks, while decreasing the production of RCRs (Matsushita et al 1998; Xiao et al 1998).

rAAV vectors are capable of infecting brain, haematopoietic stem cells, muscle, neurons and liver cells both in vitro and in vivo (Kaplit et al 1994; Zhou et al 1994). Animal studies using rAAV-mediated gene therapy have been carried out on monogenic diseases such as Fanconi anaemia, haemophilia, beta-thalassaemia, Gaucher's disease, metachromatic leukodystrophy and cystic fibrosis (Hallek and Wendtner 1996). Using an animal model of PD, Fan and colleagues successfully used two separate AAV vectors to simultaneously introduce *tyrosine hydroxylase* and aromatic *L-amino acid decarboxylase* genes into striatal neurons, resulting in improvements in behavioural deficits (Fan et al 1998). Another exciting application of rAAV vectors has been used as a treatment of Duchenne's muscular dystrophy (DMD). Previous efforts to treat DMD with viral gene therapy have been hindered by the large size (11kb) of the gene. Using a DMD mouse model, investigators delivered a series of minidystrophin genes under the control of a muscle-specific promoter (Wang et al 2000). It has been shown that the AAV vectors containing the two minigenes resulted in efficient and stable expression in the majority of myofibres and led to normal myofibre morphology, histology and cell membrane integrity.

Herpes simplex viral vectors

Herpes simplex virus (HSV) possesses a large, linear, double-stranded DNA genome (150kb). The virus infects both dividing and non-dividing cells, and does not integrate into the host genome. HSV undergoes lytic replication, but is capable of becoming latent. Latency can last for several years, after which it re-enters the lytic phase. Human pathogens HSV-1 and HSV-2 display tropism for oral and genital epithelial cells, respectively. HSV establishes a lytic infection in the peripheral epithelial cells and migrates up the sensory neurons innervating the initial lesion site. Subsequently, the infection appears to resolve. Latency is punctuated by occasional re-migration and re-infection of the virus to the periphery causing gingivostomatitis, herpes labialis, keratoconjunctivitis and genital herpes. In addition to the above manifestations, HSV-1 can produce highly lethal temporal lobe encephalitis (Roizman 1996).

Currently HSV viral vectors are produced in one of two ways. In the first approach, an amplicon system is used to generate HSV vectors by inserting a gene of interest into a plasmid that harbours an HSV origin of replication and packaging signal (Fink et al 1996). This construct is subsequently transfected into packaging cells that are infected

with a helper HSV, leading to the packaging of the amplicon into viral particles. The second method utilises viral recombination to generate the viral vector (Latchman 2001). Foreign DNA is restricted and inserted into a plasmid containing a viral gene. This shuttle construct recombines with a wild-type virus and replicates, resulting in the insertion of foreign DNA into the viral genome and inactivation of the viral genes. Recombinant viruses can then be produced and purified. HSV vectors hold great potential for gene therapy mainly due to their large cloning capacity (up to 50kb), their natural tropism for neuronal cells and the ability to generate high viral titres. However, their use as a viral vector for gene therapy will likely be restricted unless the pathogenicity associated with HSV and the risk of RCR production can be controlled and/or eliminated. As with adenoviral vectors, HSV vectors are also limited by their inability to integrate into the host genome and to produce a long-lasting transgene expression.

To eliminate the pathogenicity of HSV vectors, investigators have sought to remove the genes that mediate toxicity. Infected cell proteins (ICP), including ICP4, ICP22 and ICP27, in large part mediate the cytotoxicity of HSV (Chiocca et al 1990). Viruses devoid of ICP4 or ICP27 were no longer capable of causing encephalitis after direct injection. These elements need to be provided in packaging cell lines, raising the possibility that RCR viruses could be produced. Doubly disabled viruses have been created for safe and efficient viral production with the removal of ICP34.5 and VMW65 genes (Ace et al 1989; MacLean et al 1991). Another challenge to the use of HSV vectors concerns the cessation of gene expression once the virus enters latency. During HSV hibernation, expression of latency-associated transcripts (LAT), is driven by promoters LATP1 and LATP2. Investigations seeking to harness the long term promoting capacity of LATP1 and LATP2 have demonstrated that LATP2 promotes gene expression for longer durations (Ho and Mocarski 1988). A region of LATP2 was therefore used as an enhancer to drive viral gene expression lasting up to six months. Furthermore, LATP2 has been demonstrated to drive the expression of two genes when placed in opposite orientations, thus establishing a potential avenue to utilise the large packaging capacity provided by HSV vectors (Palmer et al 2000).

Applications of HSV-mediated gene therapy have focused predominantly on neurological diseases by exploiting HSV's natural tropism for neuronal cells. HSV-delivered nerve growth factor (NGF) helps to protect primary dorsal root

ganglion neuron cultures from oxidative damage with H₂O₂ (Burton et al 2001). Accordingly, in an animal model of diabetic cystopathy, NGF has been shown to preserve neurons exposed to chronic hyperglycaemia (Goins et al 2001). Recently, a promising application of HSV-mediated gene therapy involves intrasynaptic delivery of analgesic molecules for the treatment of chronic pain (Wilson et al 1999). Considerable effort has also been devoted to the use of HSV vectors for the treatment of PD and brain cancer. For the treatment of PD, a virus expressing both tyrosine hydroxylase and the neurotrophic factor GDNF has been constructed. This vector was shown to improve Parkinsonian behaviour and delay degeneration in a 6-hydroxydopamine animal model. In the treatment of brain cancer, particularly malignant glioma, HSV-mediated gene transfer has been used to deliver HSV-TK. When followed by the administration of the prodrug ganciclovir (GCV), the combination has been shown to have significant anti-tumour effects (Carroll et al 1997). TK phosphorylates deoxypyrimidines on GCV, yielding a defective nucleotide analogue that is toxic to actively dividing tumour cells. To further exploit this anti-tumour cytotoxicity, the connexin-43 gene has been included in the anti-tumour HSV vector (Marconi et al 2000). Gliomas express defective connexin, thereby compromising intercellular gap junctions that mediate transport of micromolecules from cell to cell. A synergistic anti-tumour effect has been observed when HSV vectors armed with HSV-TK/GCV and connexin are used. Similarly, HSV-TK vectors exert synergistic anti-tumour effects with TNF- α (Moriuchi et al 1998).

Poxvirus vectors

The poxviridae family, which includes vaccinia viruses (VV), avipoxviruses (canarypox virus), fowlpoxvirus (FPV) and many other species-specific viruses, is characterised by large brick-shaped viral particles about the size of a small bacterium (300 x 200 nm). They possess a complex internal structure and a double-stranded DNA genome (Broder and Earl 1999). The particle is wrapped with a filamentous protein component and an envelope derived from host cell membranes. Poxvirus uses its own transcriptional machinery to make transcripts in the cytoplasm. The advantage of recombinant poxviruses are their high cloning capacity for transgenes (up to 25kb) and high levels of transgene expression. However, their use has been limited by poorly defined biology, complex genome structure and potential cytopathic effects. Currently, the insertion of transgenes and the production of recombinant poxvirus are performed either

via homologous recombination or in vitro ligation (Falkner and Moss 1990; Moss 1996).

The poxvirus vectors are mainly used to generate a host immune response against cancer and infectious diseases. The immunogenicity of recombinant poxvirus has been exploited to construct live cancer vaccines expressing prostate specific antigen and carcinoembryonic antigen for treatment of prostate, colorectal, breast and lung cancers (McAneny et al 1996). They have also been used to deliver immunomodulators B7-1 and cytokines IL-2 and IL-12 to elicit a more robust immune response (McAneny et al 1996). In cervical cancer, poxvirus-mediated expression of the *E6* and *E7* genes of the human papilloma virus 16 and 18 has been used to induce tumour regression (Borysiewicz et al 1996). Finally, prime-boost vaccinations generated by poxviral vector infection have also been pursued to prevent a variety of infectious diseases (eg HIV) in which enhanced immunogenicity is achieved with the consecutive use of DNA vaccines and an attenuated virus (Ramshaw and Ramsay 2000). Ramshaw and Ramsay (2000) injected mice with a DNA vaccine against influenza haemagglutinin (HA), followed by a fowlpoxvirus (FPV) encoding the same product two weeks later. FPV is a replication deficient virus, but elicits potent cell-mediated immunity. When used in combination with a DNA vaccine, this approach leads to dramatic increases in IgG2a antibody production and clinical resistance to influenza in mice.

Other viral vectors

Aside from the viral vectors mentioned above, a number of other vectors are under development for gene therapy. Of note, the Epstein-Barr Virus (EBV), a herpesvirus with a double-stranded DNA genome, is able to infect both dividing and non-dividing cells and has a high insertional capacity (up to 150kb) compared with other recombinant viral vectors. EBV remains latent in the host nucleus as an extrachromosomal circular plasmid, resulting in long-term retention and gene expression. It has been utilised to deliver HSV-TK to hepatocellular carcinoma cell lines and animal models with successful anti-tumour cytotoxic effects (Ding et al 2001). Due to its natural tropism for B-lymphocytes, it has been used to deliver immune therapy to B-cell lymphoma tissue (Hirai et al 1997). Alpha viruses have also been developed as efficient vehicles to deliver therapeutic genes. Alpha viruses are single-stranded RNA viruses that include Semliki Forest virus and Sindbis virus. They offer alternatives for rapid and high-level gene delivery due to their high viral

titres ($>10^9$ infectious particles/ml) and their broad host range. Their natural tropism for neuronal cells has been exploited in experiments that delivered IL-12 to neural neoplasms resulting in significant tumour regression and inhibition of angiogenesis (Lundstrom et al 2001). In vivo studies are underway using alpha viruses to express the influenza haemagglutinin gene and HIV-1 *gag* gene in the production of vaccines (Caley et al 1997). In addition, efforts to engineer other viruses (eg cytomegaloviruses and foamy viruses) for gene therapy have also begun (Mocarski et al 1996; Palese et al 1996; Pandya et al 2001; Walther and Stein 2000). Although these viral vectors may have great potential as efficient gene transfer vehicles, their widespread use in gene therapy will largely hinge on improving our understanding of their biology and pathogenicity in humans, as well as developing more efficient approaches to their production.

Concluding remarks

Despite concerns regarding their pathogenicity and immunogenicity, viral vectors have proven to be a relatively safe and efficient means of gene transfer in various clinical trials. Among the commonly used vectors, retroviral (including lentiviral) vectors are more suitable for the therapies where long-term transgene expression is desired, while adenoviral vectors are ideal for studies requiring high level expression of transgenes for short durations. Adeno-associated viral vectors, on the other hand, represent a compromise between retroviral and adenoviral vectors. Poxvirus vectors are the preferred choice for vaccine-based gene therapies. HSV vectors hold great promise as vehicles to deliver therapeutic genes in the treatment of neurological diseases, but significant efforts are required to curtail their pathogenicity. Current efforts in developing alternative viral vectors will undoubtedly enrich the repertoire of gene transfer vehicles available for investigative and clinical use, although this must be premised on a thorough evaluation of their respective pathogenicity and biosafety. Taken together, the second decade of human gene therapy will bear witness to significant improvements in the efficacy of viral vector-mediated gene transfer in more clinical trials. Therefore, it is conceivable that the refinement of current vectors and/or the construction of new viral vectors will eventually lead to the development of gene therapy protocols tailored for specific diseases and/or individual patients.

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