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Importance of Viruses and Cells in Cancer Gene Therapy

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ABSTRACT

Viruses have a documented history for being used in treatment and prevention of diseases for centuries, with their application in vaccination strategies as a prime early example. In more recent history, viral vectors have been employed for gene and cell therapy of tumors. In this regard, the increased understanding of the aberrant molecular pathways underlying the process of tumorigenesis has rationalized genetic correction of these pathophysiological processes using viral vector based gene and cell therapy approaches. For example, viruses have been genetically engineered to develop oncolytic potency or mediate long-term gene expression. Also, viral vectors carrying therapeutic genes or targeting molecules have been loaded into cells, which can be exploited as delivery vehicles for these therapeutic payloads to the desired target site. However, issues pertaining to viral and cell targeting as well as host immune response elicited upon viral or cell administration remain to be addressed. In summary, the plasticity of the viral structure has rendered them amenable for the development of unique gene and cell therapy approaches, for the treatment of tumors.

Keywords: cell vehicles, immune evasion, tumor-targeting, viral vectors
Abbreviations: Δ**24**, delta-24; **AAV**, adeno-associated virus; **Ad**, adenovirus; **Ad5/H3**, Ad3 hexon protein; **Ad5/H5**, Ad5 hexon protein; **APC**, antigen presenting cells; **AuNPs**, gold nanoparticles; **CAR**, coxsackie adenovirus receptor; **CEA**, carcino-embryonic antigen; **Cox-2**, cyclooxygenase-2; **CTL**, cytotoxic T lymphocytes; **E**, early; **EGF**, epidermal growth factor; **EGFR**, epidermal growth factor receptor; **flt-1**, vascular endothelial growth factor receptor Type-1; **GCV**, gancyclovir; **HSV**, herpes simplex virus; **HSV-1**, herpes simplex virus Type-1; **HVS**, herpesvirus Samiri; **IFN**, interferons; **IgG**, immunoglobulin G; **IL-12**, interleukin-12; **MHC**, major histocompatibility complex; **MV**, measles virus; **PEG**, poly(ethylene glycol); **PKR**, RNA-activated protein kinase; **RCA**, replication competent adeno-viruses; **RGD**, arginine-glycine-aspartate; **SCC**, squamous cell carcinoma; **scDb**, single chain diabody; **scFv**, single chain antibody; **TAM**, tumor-associated macrophages; **TCR**, T cell receptor; **TIL**, tumor-infiltrating lymphocytes; **TK**, thymidine kinase; **Tregs**, regulatory T cells; **VEGF**, vascular endothelial growth factor; **VSV**, vesicular stomatitis virus

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INTRODUCTION

Viruses have been utilized for therapeutic purposes for many centuries. They are interesting biological entities harboring on the borderline between non-living things and living organisms. Upon infection of the host cells, viruses manipulate the cellular machinery to their own advantage. This ability of viruses to induce changes in the target cells presented them as one of the most suitable candidates for serving as gene therapy vectors. A variety of viral vectors has been developed for gene therapy, such as herpes simplex virus (HSV), adenovirus (Ad), adeno-associated virus

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(AAV) and measles virus (MV), just to name a few. Although viral vector-based gene therapy has demonstrated great potential for treatment of diseases like cancer, many hurdles still need to be overcome before the full potential of viral vectors can be realized.

Cell therapy describes the implantation of cells to achieve a therapeutic purpose. This definition includes routine medical procedures, such as bone marrow transplants and blood transfusions, but it also encompasses the use of genetically manipulated cells for therapeutic purposes. Gene transfer, in general, can be used to replace a mutated gene in order to restore a natural cellular function, or to confer novel therapeutic modalities to a cell. Although viral vectors are efficient gene transfer agents, as described above, systemically administered virions can be nonspecifically sequestered or inactivated via innate or acquired immune mechanisms prior to reaching the intended target cell population. However, cells can be genetically loaded using viral vectors *ex vivo* and these transduced cells can then serve as vehicles to deliver the therapeutic payload to target sites *in vivo*. The combined use of gene and cell-based medicines allows for multifaceted approaches that may be required to treat complex diseases such as cancer.

The use of viruses for gene therapy is marred with problems such as targeted delivery of the viral vector to specific cells, the immune response against the vector and the resulting toxicity issues. Attempts to resolve these issues have resulted in the development of viral vectors with improved characteristics. In this review, we discuss the strategies that have been employed for the construction of viral vectors with enhanced potential for efficacious gene therapy. We outline the construction of 'gutless' and oncolytic viral vectors, which have improvements in terms of increased transgene carrying capacity and expression, improved therapy and enhanced safety. Following this, we discuss the various approaches that have been developed for targeting viral vectors to desired cell types, as well as strategies for host immune system evasion. We end with future considerations for the utilization of viral vectors for gene therapy.

VIRAL VECTORS AND THEIR MODIFICATIONS FOR GENE THERAPY

Many viruses have been used for gene therapy. However, multiple factors limit the effective utilization of viruses for gene therapy. For instance, it has been observed that upon transgene delivery to the target cells the transgene expression diminishes with time, warranting re-administration of the viral vectors. In this regard, viral vectors utilized for gene therapy can be either integrating or non-integrating. Integrating viruses, such as retroviruses (Chang *et al.* 2001) and AAV (McCarty *et al.* 2004), integrate their genome within the genome of the host organism. Non-integrating viruses, such as adenoviruses (Marini *et al.* 2002), do not integrate into the host genome, and therefore the viral genome is lost in proliferating cells. Historically it was therefore believed that integrating viral vectors would provide long-term expression of the therapeutic gene in the host and thus would not require repeated administration, unlike the non-integrating viruses. However, pre-clinical experience with the utilization of integrating vectors such as AAV for gene therapy has demonstrated that repeated administration might be necessary for integrating viruses as well. For example, when AAV was used for genetic correction of a cystic fibrosis defect in the lungs, the limited viral transduction efficiency resulted in low therapeutic gene delivery to the lung cells. Moreover, an antibody response generated against the viral vector reduced the amount of gene transfer that could be achieved and also prevented re-administration of the virus (Halbert *et al.* 2000). Modification of viral vectors to circumvent or mitigate an immune response against the infected cell and the vector itself is thus warranted, even if integrating vectors are used.

Gutless vectors

As noted above, administration of viral vectors results in an immune response. Upon first vector administration, the body responds by mounting an immune response against the virus itself, viral proteins that are expressed in the infected cells and the therapeutic gene if it is foreign to the host. This immune response severely limits the efficacy of the therapeutic vector since infected cells that express the transgene will be cleared from the body. In addition, the development of immunological memory restricts the efficacy of subsequent administrations, and limits the dosage and the number of times the viral vector can be administered to the patient. To circumvent the immune response generated against the viral vector and the viral proteins, one of the strategies employed is the deletion of the unnecessary viral genome sequences. The removal of the unnecessary viral genome sequences drastically reduces the immunogenicity of the viral vector, and increases the efficacy of viral gene therapy. Another benefit of the deletion of viral genome sequences is the increase in carrying capacity for foreign therapeutic genes. This is especially important when large genomic sequences need to be delivered, such as the dystrophin gene for the treatment of Duchenne muscular dystrophy (Bogdanovich *et al.* 2004).

As a representative example of viral vectors with deleted genome sequences, the construction of adenoviral (Ad) vectors carrying progressively less amounts of the viral genome can be studied, which is described below.

First generation Ad vectors

One of the considerations in deciding which viral genes can be deleted from the genome is the role played by these various genes in the viral reproduction cycle. As an example, for Ads it was discovered that early (E) expression gene products could be provided *in trans* in order to achieve mature adenoviral progeny production during the production process. In particular, E1, E2, E3 and E4 regions have been deleted or inactivated. Initially, it was the E1 region that was deleted from the Ad genome considering its essential role in transcriptional activation of other early genes, inhibition of apoptosis of the infected cell and modification of the intracellular environment to make it more conducive for Ad protein production (Akusjarvi 1993; Flint *et al.* 1997; Young *et al.* 1997; Dyson 1998). Deletion of E1 resulted in replication deficient viral vectors that were propagated in helper cell lines that provided E1 gene product *in trans* (Trapnell *et al.* 1994).

Subsequently, the E3 region was deleted, which encodes proteins that inhibit various death pathways elicited by the host immune system against the cells infected with Ad vectors (Wold *et al.* 1995, 1999). The Ad vectors with deleted E1, with or without deletion of E3, are referred to as 'first generation' Ad vectors (**Fig. 1**). The first generation Ad vectors have a carrying capacity of ~8 kb for foreign genes (Bett *et al.* 1993). However, unfortunately, even after deletion of E1 and E3, these viral vectors still suffer from immune resistance due to leaky viral protein expression in the host. This results in clearance of the viral vectors as well as host cells infected with the virus (Yang *et al.* 1994). In addition, propagation of these vectors in complementing cell lines may result in replication competent adenoviruses (RCA) due to recombination with the viral DNA sequences present in the complementing cell line (Amalfitano *et al.* 1998). The RCA contaminates the replication incompetent viral vector preparations. The possibility of uncontrolled replication of this RCA contaminant in the patient increases the safety considerations.

Second generation Ad vectors

The problems with the first generation Ads mentioned above sparked the further minimalization of the viral genome, and thus the viral protein expression in the host. For

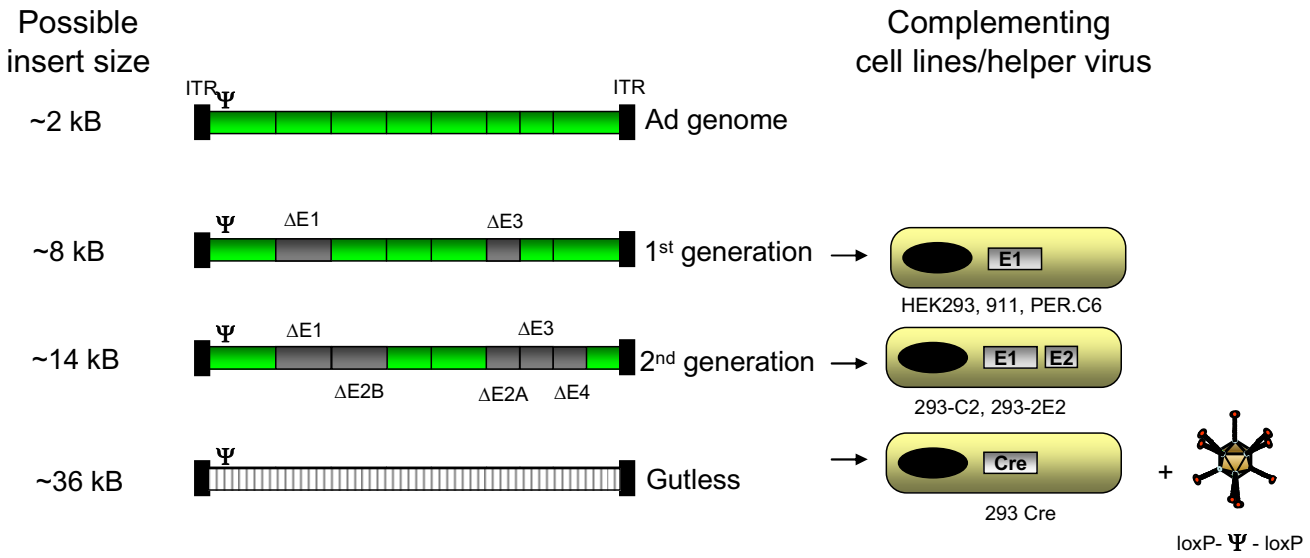

Fig. 1 Diagram of viral genomes corresponding to the wild type Ad genome and three generations of Ad vectors. Deleted genes are shown in gray. The function of the deleted genes is delivered *in trans* by complementing cell lines or a helper virus. Each generation has tolerated larger insert sizes, culminating in gutless vectors that can package inserts up to 36kB. An example of how these gutless vectors are produced is the use of a helper virus that incorporates loxP sites that flank the packaging signal (ψ) in its genome. When this virus infects cells that express the Cre recombinase and are transfected with the gutless genome, the packaging signal will be deleted from the helper virus genome that will thus not be incorporated into the new virions, resulting instead in packaging of the gutless genome that does have the packaging signal.

this, in addition to E1 and E3, the E2 region was also deleted (Amalfitano et al. 1998). The E2 region encodes proteins needed for Ad DNA replication (van der Vliet 1995). Following the E2 deletion, the E4 region was also deleted. The E4 region encodes multiple proteins that are utilized for Ad DNA replication, mRNA transport and splicing, inhibition of host cell protein synthesis, and regulation of apoptosis (Bridge *et al.* 1989; Huang *et al.* 1989). With regards to E4, viral vectors with modifications other than deletion, such as removal of the E4 promoter, have also been generated. The vectors with deletions in E2 and E4, along with E1 and E3 in different combinations, are referred to as 'second generation' Ad vectors (**Fig. 1**). This second generation has a transgene carrying capacity of ~14 kb (Alba *et al.* 2005).

In addition to reducing the host immune response and increasing the transgene carrying capacity of Ad vectors, these deletions also resulted in more severely crippled replication deficient vectors than the first generation vectors, thereby increasing their safety profile (Parks *et al.* 1996). For example, an Ad vector carrying the tumor suppressor p53 in the deleted E1 region, deleted for E3 and having an inactivated E4 region was compared to a vector with a wild type E4 region, to analyze whether deleting multiple viral genes can enhance the safety profile of the Ad vector. The Ad vector with the inactivated E4 region demonstrated a reduced host immune response compared to the control vector, resulting in reduced toxicity and prolonged duration of p53 expression *in vivo* in immunocompetent mice (Ji *et al.* 1999).

However, despite these encouraging results, the residual gene expression from the remaining viral genes still resulted in immunogenicity and toxicity for these second generation Ad vectors. In this regard, it was soon realized that for the Ad vectors, in addition to the early region genes, many more genes could be deleted and their functions provided *in trans*. Thus, true "gutless" vectors came into being.

Third generation 'gutless' Ad vectors

Gutless vectors are the most advanced form of Ad vectors currently available. These vectors are devoid of all the viral genes except those that are required *in cis* for packaging and replication. These vectors are also known as gutted, amplicon, high-capacity, helper-dependent and fully-deleted adenoviral vectors (**Fig. 1**). The transgene carrying capacity of gutless vectors is ~36 kb (Alba *et al*. 2005). These vectors have demonstrated a better safety profile than the first and second generation of Ad vectors. However, there are still some problems with gutless Ad, especially in regard to problematic production of high titers that are required for clinical use. Also, contamination with RCA remains a concern that requires further investigation (Alba *et al*. 2005). These problems are currently being countered utilizing various approaches, such as episomally maintained Ad vectors (Kreppel *et al.* 2004) and improved packaging cell lines (Sakhuja *et al.* 2003; Alba *et al*. 2005).

In addition to the above mentioned 'gutless' Ad vectors, other viral vectors with deleted viral genomes have been constructed. For example, lentiviral (Naldini *et al.* 2000) and retroviral vectors devoid of viral genome sequences in the transfer vector have been constructed, such that no viral proteins are produced in the infected cells.

In conclusion, even though many issues pertaining to efficient production of the gutless vectors still need to be resolved, it is anticipated that gutless vectors will be increasingly used for gene therapy in coming years due to their improved efficacy and safety profile.

Oncolytic viral vectors

The proposed use of viruses for gene therapy applications has always caused concern because of the inherent pathogenic nature of these agents. In this regard, viral vectors were modified to limit their replication potential in the host organism (**Fig. 2**). Therefore, initially only the gene delivery capacity of viral vectors was utilized for gene therapy. Although this addressed the concerns related to safety issues in a cancer therapy context, this also prevented the use of a very efficient cell killing method, i.e., viral vector mediated lysis of infected tumor cells. For example, replication deficient Ad vectors were utilized to deliver a bacterial cytosine deaminase gene into glioma cells, which chemosensitizes glioma cells for otherwise non-toxic 5-fluorocytosine (Dong *et al.* 1996). This strategy kills those tumor cells which are infected with the viral vectors, but not the remaining tumor cells. However, if the viral vector could replicate selectively in the tumor cells thereby resulting in oncolysis, then the viral progeny could potentially infect

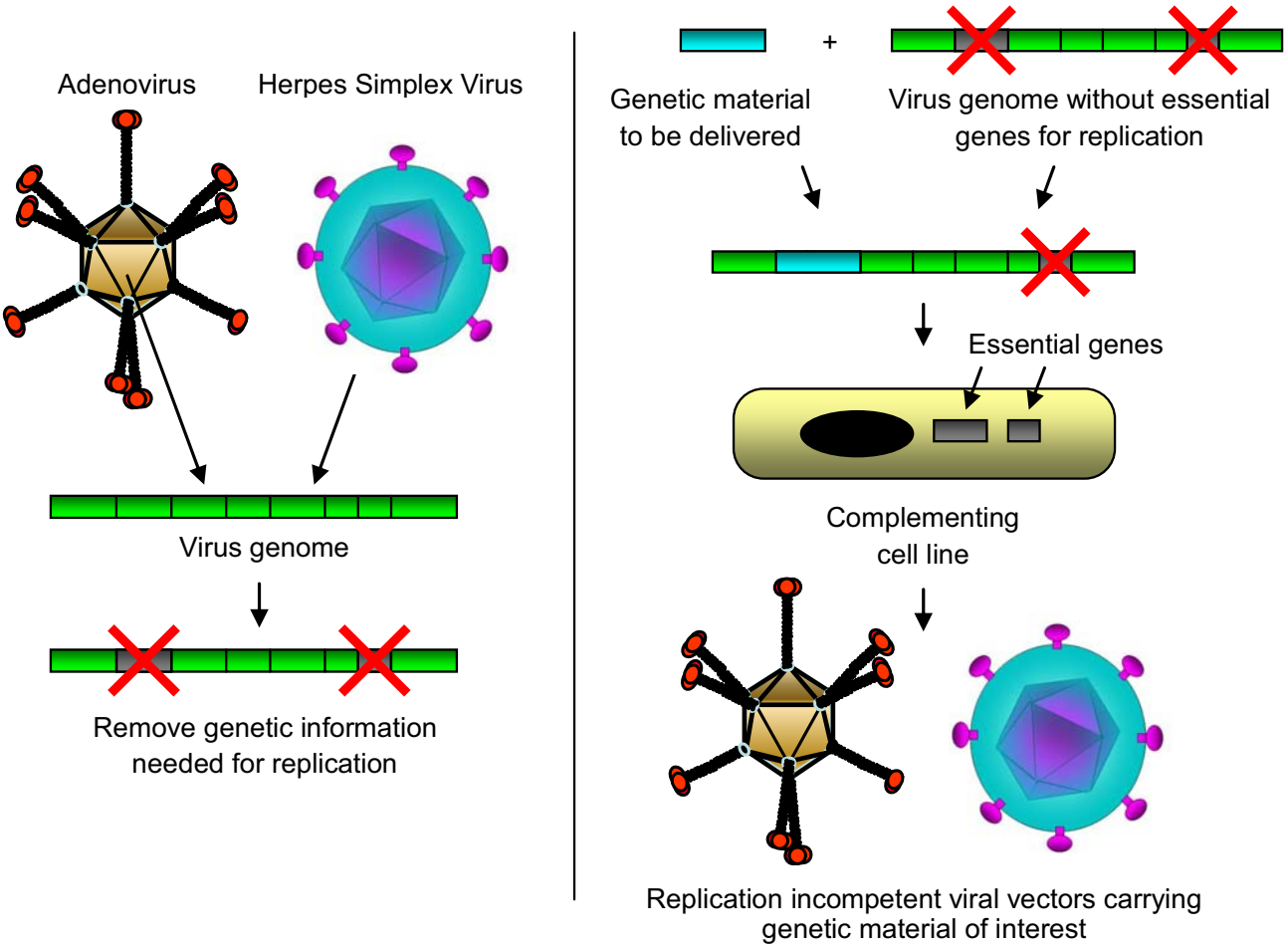

Fig. 2 Modification of replicating viruses into non-replicating gene therapy vectors. Left: Adenovirus and Herpes Simplex Virus are examples of viruses that can be modified into replication incompetent gene therapy vectors by deleting the genes necessary for viral replication (gray rectangles) from the viral genome (green rectangles). Right: The deletion of genes essential for viral replication provides space for therapeutic genes of interest (blue rectangle), which can be incorporated into the genome. For vector production, the gene products necessary for viral replication (gray rectangles) are provided *in trans* in a complementing cell line, resulting in replication incompetent vectors that carry the therapeutic gene of interest.

the adjoining tumor mass that escaped the primary infection. Moreover, replicative virus can kill tumors in combination with the chemosensitizing approach.

Thus, in order to utilize the inherent cell killing potential of viruses with a lytic replication cycle but avoid side-effects in healthy cells, viral vectors capable of selective replication in tumor cells were constructed. These viral vectors are replication competent and thus oncolytic, but only in target cells by using a variety of mechanisms, as will be described below. The use of oncolytic viruses for killing target tumor cells has been defined as virotherapy (Nettelbeck *et al.* 2003).

Advantages of oncolytic viral vectors

There are multiple advantages that mandate the use of conditionally replicative oncolytic viruses for tumor treatment. Being replicative, after the initial infection, viral progeny can spread through the tumor mass and effectively remove all of the tumor cells. In addition to their oncolytic properties, these viruses can also introduce therapeutic genes, such as suicide genes and cytokines. In addition, expression of viral proteins can be utilized to elicit an anti-tumor immune response, increasing the effectiveness of tumor treatment.

A variety of oncolytic viruses have been used as potential candidates for oncolytic therapy, including Herpes Simplex Virus (HSV), reovirus, vesicular stomatitis virus (VSV), and Ad, to name a few.

The viruses currently under investigation for oncolytic therapy are either inherently selective or are genetically modified to be selective for replication competence in tumor cells. In this regard, herpesvirus samiri (HVS) was demonstrated to be naturally selectively oncolytic for the pancreatic cancer line PANC-1 (Stevenson *et al.* 2000). Similarly, human reovirus (Hashiro *et al.* 1977) and VSV (Stojdl *et al.* 2000) were shown to replicate more efficiently in transformed cell lines as compared to non-transformed cells lines (Ring 2002). Reovirus is an example of a naturally oncolytic virus with replication limited to tumor cells with an activated Ras-signaling pathway. Upon infection of normal cells by reovirus, the early viral transcripts activate double-stranded RNA-activated protein kinase (PKR), which inhibits viral protein translation and viral replication. However, in tumor cells, the activated Ras as well as upstream and downstream elements of the Ras-pathway, inhibit (or reverse) PKR activation, thereby allowing reoviral replication resulting in oncolysis (Wilcox *et al.* 2001). The activating mutations in *Ras* have been reported for >30% of tumors. In addition, the mutations in upstream and downstream arms leading to constitutive Ras pathway signaling have been reported for an even greater proportion of tumors (Norman *et al.* 2004). Based on these facts, reovirus has been shown to be effective as an oncolytic agent for a variety of tumors, including malignant glioma (Wilcox *et al.* 2001), breast cancer (Norman *et al.* 2002) and pancreatic cancer (Etoh *et al.* 2003) in animal models.

VSV provides an example of an oncolytic virus where a tumor cell advantage over normal cells has been exploited for selective viral oncolytic activity. All cells exposed to viral infection produce antiviral interferons (IFNs). However, cancer-specific mutations of gene products in the IFN pathway have been reported in tumors (Stojdl *et al.* 2000).

This defect in IFN response against viral infection has been utilized for selective VSV replication and oncolysis of tumors, such as melanoma (Stojdl *et al*. 2000) and colorectal carcinoma metastatic to liver (Shinozaki *et al.* 2005) in mouse models.

In some cases, natural oncolytic activity has been artificially restricted to a particular type of cell, thereby rendering the virus useful for selective treatment of tumors. For example, oncolytic herpes simples virus type 1 (HSV-1) has been exploited for tumor therapy because it can be modified for restricted viral replication in proliferating glioma cells. Of note, one of the advantages of HSV-based oncolytic vectors is the potential use of the antiviral drug acyclovir, should replication become out of control. HSV-1 based vectors have been tested in various phases of clinical trials for glioma with promising results. In addition, oncolytic viral activity of HSV-1 has been combined with the elicitation of an anti-tumor immune response, in order to improve tumor treatment. For example, Wong *et al.* used an oncolytic HSV-1 expressing the pro-inflammatory cytokine IL-12 for treatment of distantly metastatic squamous cell carcinoma (SCC), and observed significantly improved survival in mice with this combination of oncolytic and immune therapy (Wong *et al.* 2004) as compared to oncolytic therapy alone for treating disseminated disease.

A similar strategy based upon a combination of oncolysis and immunomodulation was used with an oncolytic recombinant VSV expressing murine IL-12 (rVSV-IL12). This virus demonstrated a significant reduction in murine squamous cell carcinoma volume as compared to the control virus without IL-12 (Shin *et al.* 2007).

In addition to above listed viruses, conditionally replicative oncolytic adenoviruses (CRAds) have been used for tumor treatment. These vectors have been developed based upon the understanding of aberrant molecular pathways in tumor cells in conjunction with the understanding of Ad biology. For example, the Rb and p53 oncogenes have mutations in many tumors. This fact has been exploited for the generation of an oncolytic Ad vector, delta-24 (Δ24). In this vector, the E1A region that interacts with Rb has been deleted. This virus therefore replicates more efficiently in tumor cells with mutations in Rb as compared to healthy cells (Fueyo *et al.* 2000). Similarly, another Ad genome sequence, E1B 55kDa, which interacts with p53, was deleted to construct a CRAd named *dl*1520 (Onyx-015) (Bischoff *et al.* 1996). This virus replicates in tumors with mutations in p53. However, it is now assumed that in addition to p53, other factors like infectivity and cell permissiveness also contribute to the differential replication of Onyx-015 (Ring 2002). It was determined that the use of Onyx-015 along with chemotherapy might have synergistic effects for tumor treatment (Khuri *et al.* 2000). However, Onyx-015 is not suitable by itself due to limited replication potency. One of the reasons for the limited efficacy of Onyx-015 might be the loss of functions of E1B that are critical for the Ad life cycle, such as mRNA transport and shut-off of host cell protein synthesis (Ring 2002).

Another type of CRAds are those with tissue specific promoters to impose transcriptional limitations for oncolytic replication in specific target cells. For example, cyclooxygenase-2 (Cox-2) has been shown to be highly expressed in a number of epithelial tumors (Lam *et al.* 2007). Based on this consideration, an infectivity enhanced CRAd with the E1 region under transcriptional control of the Cox-2 promoter was constructed. This vector demonstrated potent anti-tumor effects as compared to the wild type vector for pancreatic (Yamamoto *et al.* 2003) and ovarian tumors (Kanerva *et al.* 2004) both *in vitro* and *in vivo*. Another example of transcriptional control of CRAd replication exploits the fact that tumor cell growth is dependent upon neovasularization. For this purpose, vascular endothelial growth factor (VEGF) is produced by tumor cells to drive the angiogenesis. Takayama *et al.* utilized a tropism-modified CRAd in which expression of E1 region, necessary for viral replication, was put under transcriptional control of VEGF promoter. This vector replicated efficiently in lung tumors *in vitro* and *in vivo* (Takayama *et al.* 2007).

Issues pertaining to oncolytic viral therapy

Despite all these developments, many problems have hampered successful utilization of oncolytic viruses for tumor treatment. Upon intra-tumoral or peripheral administration of the oncolytic virus, it was expected that viral progeny would spread to the entire tumor mass and eliminate the tumors efficiently. However, when the first pre-clinical analyses were performed, it was apparent that oncolytic viruses did not spread through the tumor mass as expected. This might be due to the large size of the virus (90 nm for Ad), and physical barriers such as cell-to-cell barriers, basement membranes, necrotic regions and intermixed normal cells (Vile *et al.* 2002).

Another issue that needs to be addressed is the targeting of the virus to specific cells. For example, Ad vectors bind to the coxsackie adenovirus receptor (CAR), which is expressed at high levels in normal tissues of the body such as liver, but at low or negligible level in certain tumors. This results in low viral vector infection efficiency for the tumor cells. In order to achieve the needed infectivity enhancement, viral vectors have been genetically modified. For instance, Krasnykh *et al.* constructed chimeric Ad5/3 vectors, in which the knob domain of Ad5 was replaced by the knob domain of Ad3. This chimeric virus was shown to bind to cells by utilizing receptors other than CAR (Krasnykh *et al.* 1996), resulting in its ability to infect cell lines deficient in CAR-expression. Another example for the Ad vector infectivity enhancement is provided by Wu *et al.*, who constructed Ad vectors with RGD and pK7 motifs in the fiber. It is known that the amino acid sequence arginine-glycine-aspartate (RGD) binds to integrins. Furthermore, it has been demonstrated that poly-lysine sequences (pK7) bind to heparin sulfate-containing receptors. Integrins and heparin sulfate-containing receptors are overexpressed in many tumors. The double modified Ad vector containing RGD and pK7 motifs in the fiber was shown to be capable of infection in both CAR-positive as well as CAR-negative cell lines. The observed infectivity enhancement was a result of the utilization of additional receptors for cell entry by the double modified Ad vectors (Wu *et al.* 2002b).

In addition to the above issues, it has been realized that oncolytic potency of the viral vectors must be determined before these vectors are employed in clinical trials. The oncolytic vectors are usually evaluated in immunodeficient mouse models containing xenografts of human tumors. However, being immunodeficient, these mouse models do not represent the actual scenario in the body of an immunocompetent human patient. In addition, mouse tissues are not very permissive for the replication of human viral vectors such as Ad vectors. In order to overcome these issues, Thomas *et al.* have developed a Syrian hamster model for study of the oncolytic Ad vectors. This model is immunocompetent and permissive to infection by the Ad vectors, thereby mimicking the human physiological system more closely than the mouse models (Thomas *et al.* 2006). However, this model still needs better characterization before its potential can be fully exploited.

In addition to the use of animal models, liver and tumor tissue slices from patients have also been used to evaluate the toxicity characteristics of oncolytic viruses. Since tissue slices can be directly derived from cancer patients, they provide a more physiologically relevant platform for analysis of toxicity of oncolytic viruses (Stoff-Khalili *et al.* 2007b). However, there are practical considerations regarding the availability of fresh tissue slices that are currently limiting their widespread application.

Another method to analyze the characteristics of oncolytic viruses is the use of *in vitro* human cell cultures. However, adherent cell culture is a two-dimensional system as opposed to the three-dimensional tumor environment. Thus,

novel assay systems are being developed to aid in pre-clinical analysis of the oncolytic potency of the viruses. For example, Lam *et al.* have developed a tumor-spheroid three-dimensional system as compared to two-dimensional cell culture mono-layers to measure the viral penetration and oncolytic potency (Lam *et al*. 2007).

Thus, selectively replicative oncolytic viruses are a potent tool for treatment of diseases like cancer. These viruses will be used more widely for treatment once issues related to their oncolytic potency and safety are resolved.

TARGETING OF VIRAL VECTORS

In gene therapy, it is imperative that the therapeutic gene is delivered specifically to the intended target cells. Similarly, the viral vectors that are used for oncolytic therapy must infect and replicate only in the particular cell type that needs to be killed. However, the native tropism of viruses utilized for gene therapy does not necessarily correspond with the desired cell type that needs to be infected. For example, Ads bind to CAR, which is expressed at high levels in normal tissues of the body, such as liver, and not in the intended targets like tumor cells. Therefore, upon Ad vector administration, liver related toxicity can be observed. Similarly, retroviruses are known to infect proliferating cells. Although tumor cells proliferate rapidly, there are other body cells that also undergo proliferation. Thus, retroviral replication must be restricted to tumor cells only and not to normal body cells. Another example is AAV-2, which infects liver cells. This interaction is mediated by heparin sulfate proteoglycan molecules that are present on liver cells. Thus, to use AAV-2 for gene therapy of extrahepatic tissues, its binding to hepatic cells must be perturbed. Therefore, for the development of effective gene therapy viral vectors, the native viral tropism needs to be ablated and viral vectors need to be retargeted to tumor cells.

The targeting of viral vectors can be either transductional or transcriptional. Transductional targeting involves modification of viral tropism whereas transcriptional targeting involves modulation of the viral gene expression such that viral genes are expressed only in desired cell types.

Transductional targeting

Transductional targeting has been achieved through a variety of approaches, including bifunctional adapters and genetic modifications of the viral vector.

Bifunctional adapters for transductional targeting

Bifunctional adapters, as the name indicates, are a combination of two different subunits, one of which binds to the viral vector and the other binds to the target cell. The two different subunits can be attached to each other by either chemical or genetic methods. There are a variety of subunits, some of which will be discussed in more detail below.

Chemically conjugated bifunctional adapters

Due to the technical ease of coupling two subunits by chemical methods, the initial bifunctional adapters contained subunits that were chemically linked. For example, a chemically coupled bispecific antibody conjugate was generated, in which an antibody against Ad was chemically liked to an antibody against epidermal growth factor receptor (anti-EGFR). This bispecific antibody was successfully utilized for targeting Ad vectors to EGFR expressing human glioma cells (Miller *et al.* 1998). However, due to the chemical coupling strategy employed for linking the two subunits, there was variability in the resulting bispecific antibody product, leading to batch to batch variations. Thus, a more consistent production strategy was desired.

Genetically conjugated bifunctional adapters

To circumvent the problems observed with chemical coupling of the subunits, genetic coupling of the subunits constituting the bifunctional adapters was endeavored. For example, an adenobody is a genetic fusion of a single chain antibody (scFv) directed against the Ad fiber knob to a ligand that binds to a target cell. For example, Watkins *et al.* fused a scFv against Ad knob with epidermal growth factor (EGF), which can bind to EGFR on human cells (Watkins *et al.* 1997). Haisma *et al*. further extended the adenobody approach by constructing a bispecific scFv, called a single chain diabody (scDb). For this purpose, a scFv against Ad was genetically fused with a scFV against the EGFR (Haisma *et al.* 2000). Another example of a scDb is for melanoma retargeted Ad vectors, where a scFv against Ad was genetically fused with a scFv against the high molecular weight melanoma antigen (Nettelbeck *et al.* 2004).

In addition to the use of scFc against the Ad knob, other types of subunits with an affinity for Ad knob have been utilized for construction of bifunctional adapters. For example, the ectodomain of the native adenoviral receptor CAR fused to scFvs that target tumor associated antigens has also been exploited for retargeting Ad vectors to specific cells. In this regard, Everts *et al.* fused the ectodomain of CAR, sCAR, with a scFv directed against carcino-embryonic antigen (CEA), which is over-expressed in the adenocarcinomas of the gastrointestinal tract, lung and breast. This bifunctional adapter successfully re-targeted Ad vectors to CEA artificially expressed in the lungs after intravenous administration (Everts *et al.* 2005).

Using these bifunctional adapters, Ad vectors have been efficiently retargeted to desired cells or tissues. In addition, the retargeting and accompanying ablation of native tropism also reduced the Ad vector sequestration in liver, leading to reduced toxicity. However, binding a bifunctional adapter to the viral vector requires an incubation step before infection can be achieved. In addition, even though genetic bifunctional adapter molecules themselves are of a homogenous nature, the incubation of them with Ad vectors will still result in batch-to-batch variations, which are undesirable for clinical application. Moreover, there is always a possibility that the bifunctional adapter does not attach to all the viral sites, thereby sustaining the possibility of viral infection in unintended target cells. In order to resolve these issues, genetic transductional targeting approaches have been developed.

Genetic transductional targeting

A variety of vectors and methods have been used to genetically modify viral vectors in order to achieve the required targeting. For example, Girod *et al.* inserted a 14-amino-acid targeting peptide, L14, into the capsid of AAV-2. The resulting capsid modified virus was demonstrated to efficiently infect previously resistant cell lines that display the integrin receptor recognized by L14 (Girod *et al.* 1999). Although insertion of a targeting moiety against a particular target cell receptor into the viral capsid is an efficient way of targeting the virus, it is very time consuming to incorporate a specific targeting ligand into the viral capsid for a cell type of interest. Thus, a more general targeting approach might be more beneficial, especially for screening purposes. In this regard, Ried *et al.* incorporated the immunoglobulin G (IgG) binding domain of protein A, Z34C, into the AAV-2 capsid. The resulting AAV-2 mutants could be targeted to distinct hematopoietic cell lines using an antibody against CD29 (β_1-integrin), CD117 (c-kit receptor) and CXCR4 (Ried *et al.* 2002). Another example of a general targeting approach is provided by genetically modified Ad vectors. In this regard, Noureddinni *et al.* also fused the Fc-binding domain of *Staphylococcus aureus* protein A into a chimeric fiber expressed on Ad vectors. This modified Ad vector can now be utilized to infect a broad range of target cells, depending on the monoclonal antibody that is coupled to the Fc-binding domain on the Ad vector

(Noureddini *et al.* 2006).

In addition to genetically incorporating the targeting ligands in the capsid of the viral vectors, another approach that has been proposed is pseudotyping. It involves substituting the receptor binding proteins of one virus for those of another virus. For example, an AAV-2 genome encapsidated into a parvovirus B19 capsid can provide a new tool for AAV-2 targeting to specific cells, based on the natural tropism for human erythroid progenitor cells of parvovirus B19 (Ponnazhagan *et al.* 1998).

One of the most advanced forms of genetic transductional targeting is to directly incorporate antibodies recognizing the target cell antigens into the viral capsid. This has recently been achieved for Ad vectors. Hedley *et al.* genetically incorporated a scFv into the fiber of Ad vectors and demonstrated successful targeting to receptors on the surface of target cells (Hedley *et al.* 2006). It will be of interest to see the targeting capacity of these genetically modified vectors in an *in vivo* context, and determine their translational potential.

Similar genetic approaches have also been applied for targeting of other viruses. For example, scFv against CD38 and EGFR have been genetically incorporated into measles virus (MV) (Nakamura *et al.* 2005). More recently, Hasegawa *et al.* genetically modified the tropism of MV for targeted virotherapy of ovarian cancer. For this purpose, they incorporated the scFv specific for α-folate receptor (FRα), which is over-expressed on 90% of nonmucinous ovarian cancer, into the attachment protein of MV. This virus reduced the tumor volume and also increased the overall survival of mice as much as the parental virus, but without the side effects of the untargeted virus (Hasegawa *et al.* 2006).

Transcriptional targeting in combination with transductional targeting

The above examples illustrate the approaches that have been developed for targeting viral vectors to specific cells. However, a strategy to supplement the tranductional targeting is to involve transcriptional targeting as well. For this purpose, cell specific promoter elements have been incorporated into the genome of viral vectors to limit viral gene expression in specific cell types. For example, Muller *et al.* used AAV-2 devoid of binding to their primary receptor heparin sulfate proteoglycan. In this virus, they incorporated a luciferase reporter gene under the control of 1.5-kb cardiac myosin light chain promoter, fused to the cytomegalovirus immediate early enhancer. The combined transductional and transcriptional targeting with this virus resulted in efficient gene transfer to cardiac cells *in vivo* and also had a significantly reduced hepatic sequestration (Muller *et al.* 2006).

Another example for combined transductional and transcriptional targeting is provided by Ad vector targeting to endothelial cells. To achieve this targeting, Reynolds *et al.* utilized a chemically linked bifunctional adapter. For this, a Fab fragment against Ad knob was chemically coupled to an antibody against angiotensin converting enzyme (9B9), which is a membrane-bound ectopeptidase expressed on pulmonary vascular endothelium. For transcriptional targeting, the promoter for vascular endothelial growth factor receptor type-1 (flt-1), which has high activity in endothelial cells, was utilized to drive the expression of a luciferase reporter gene. The combined transductional and transcriptional approaches resulted in a synergistic 300,000-fold improvement in the selectivity of transgene expression for lungs as compared to the liver, which is the usual vector sequestration site (Reynolds *et al.* 2001). Thus, combined targeting approaches have been shown to be useful for cell type specific viral vector delivery and therapeutic gene expression, for improved gene therapy.

Targeting of the viral vectors to the appropriate cells is crucial for development of an efficient gene therapy regimen and as illustrated by above examples, many unique strategies have been developed for this purpose. Though specific target cell delivery increases the therapeutic gene transfer to target cells, unfortunately an immune response elicited against the viral vector still limits full utilization of targeting approaches.

STRATEGIES FOR IMMUNE SYSTEM EVASION BY VIRAL VECTORS

Viral vectors utilized for gene therapy are recognized as foreign by the host in which they are injected, and are therefore countered by an immune response. The immune response consists of innate and adaptive responses. The innate response is elicited upon recognition of the foreign viral capsid components by the immune system. The innate response leads to clearance of the viral vector before the viruses have had a chance for primary infection (Bessis *et al.* 2004; Muruve 2004). This diminishes the efficiency of the transgene delivery to target host cells. Following successful viral infection of host cells, the adaptive arm of the host immune system is activated against the viral proteins that are produced in the host cells and the therapeutic gene if it is foreign to the host. The adaptive response also results in the development of immune memory, which further limits viral re-administration (Bessis *et al*. 2004). Also, pre-existing immunity against the viral vector further compounds the problem of efficient therapeutic transgene delivery by the viral vector. For example, Ads are one of the causative agents of the "common cold" and thus, many patients have pre-existing humoral immunity against the viral vector. This leads to rapid clearance of the therapeutic viral vector from the blood stream, prevents re-administration of the viral vector and results in overall reduction in the efficacy of the viral vector based gene therapy. This suggests that suppression or avoidance of the immune system would be needed to achieve sufficient viral vector based therapeutic effects. However, the immune response generated against the viral vector and/or the delivered transgene can also be exploited for manipulating the host immune system in developing an effective immune response against tumor cells. The following examples illustrate these points in more detail.

Immuno-suppression

To circumvent the immune system mediated removal of the viral vector, a variety of approaches have been developed. In this regard, immuno-suppressants have been used to blunt the immune system of the host, thereby increasing the transgene delivery and expression by the viral vector. For example, Jooss *et al.* administered an Ad vector along with different doses of cyclophosphamide, which suppresses T cells. They demonstrated an effective blockade of both T and B cell responses in the liver and the lungs of C7BL/6 mice using this strategy. This resulted in prolonged transgene expression, reduced inflammation and allowed re-administration of the Ad vector (Jooss *et al.* 1996). However, the use of immunosuppressive drugs, which diminish the immune response capacity of the patient against foreign pathogens, causes concern.

Another strategy that has been utilized for immune system modulation involves perturbation of the host immune system at the level of cross-talk among different immune cell types. Disruption of the co-stimulatory interactions between antigen presenting cells (APCs) and B and T cells has been shown to be successful for reducing the cellular as well as humoral response generated against the viral vector. APCs present processed foreign antigens in association with major histocompatibilty complex (MHC) molecules to T cells for their activation. In addition to the antigenic peptide and MHC interaction with the T cell receptor (TCR), other co-stimulatory molecules also play an important role in T cell activation. In this regard, B7 proteins on APCs bind to CD28 on T cells, providing a critical second co-stimulatory signal, especially for the primary response

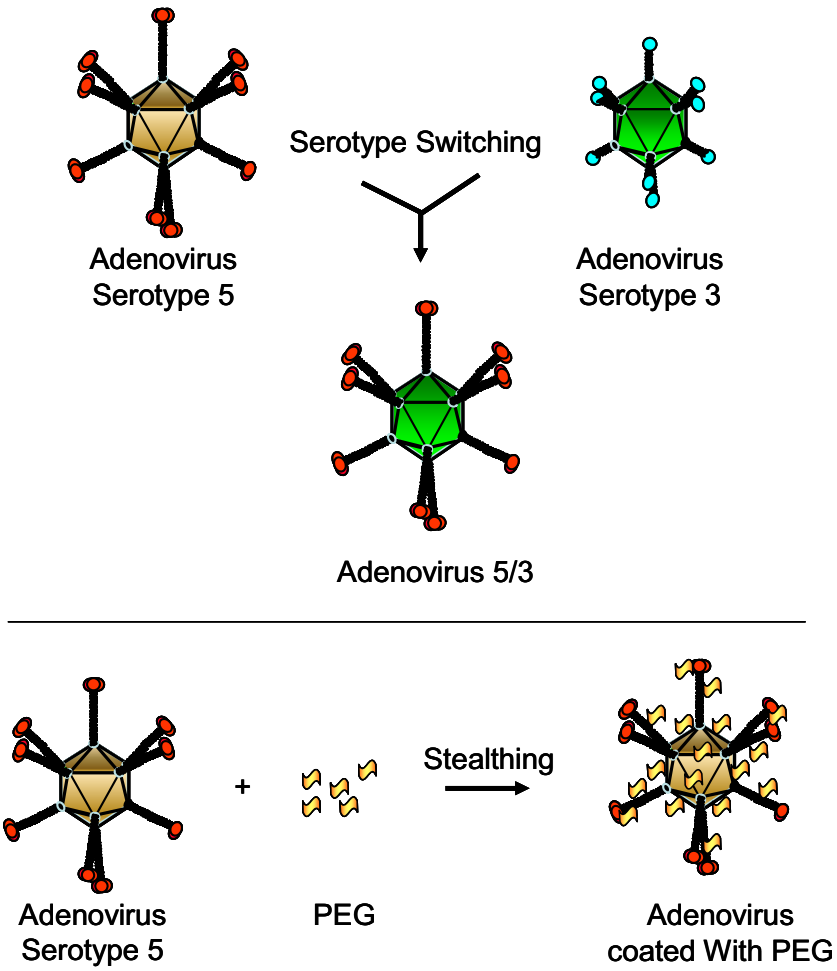

Fig. 3 Strategies employed for immune system evasion. Pre-existing immunity against gene therapy vectors is a major limitation to effective gene transfer. Strategies to overcome this hurdle include serotype switching (top) and physical masking of antigenic epitopes (bottom). Top: Serotype switching encompasses the construction of vectors containing capsid proteins from different serotypes. For example, a chimeric Ad5 vector expressing hexon protein of Ad3 was constructed. This vector was not recognized by antibodies against the hexon protein of Ad5, thereby allowing vector re-administration. Bottom: Alternative strategies have included physical masking of antigenic epitopes on viral vectors. For example, poly(ethylene glycol) molecules can be chemically conjugated to Ad vectors, which protect the vectors against antibody recognition.

of the naïve T cells to novel antigens. B7 also binds to CTLA4 on the T cell surface, which primarily dampens T cell activation. Thus, blocking the interaction of B7 with CD28 will inhibit T cell priming, which will inhibit downstream immune responses activated by T cells. In this regard, it has been shown that the extracellular domain of CTLA4 fused to an immunoglobulin IgGFc domain (CTLA4Ig) binds to B7 with 20-fold higher affinity as compared to CD28. A consequence of the interaction of antigen-MHC with TCR in the absence of B7-CD28 interaction can be the induction of T cell energy or prolonged unresponsiveness (Kay *et al.* 1997).

Another immune system interaction that has been disrupted is the interaction between activated T cells and B cells. Activated T cells express CD40, which binds to CD40 ligand on the surface of B cells, which is critical for the development of a humoral B cell response. This interaction can be blocked by a monoclonal antibody, MR-1, against CD40 ligand. Blockade of this interaction results in immunodeficiency in antibody response (Kay *et al*. 1997). A combination of CTLA4Ig with MR-1 has been utilized for suppressing the host immune system. For example, it has been shown that administration of MR-1 protein along with CTLA4Ig allowed for re-administration of AAV in lung (Halbert *et al.* 1998) and Ad in the liver (Kay *et al.* 1997).

An alternate strategy that has been utilized for immunosuppression is incorporation of immune system suppressor genes in the viral vector itself. Immune system suppressing genes have been used to blunt the immune response even when the viral vector encoded proteins are produced in the host cells. For instance, Haralambieva *et al.* incorporated the P gene from a wild type measles virus (MV) strain into an oncolytic MV. The P gene product inhibits interferon (IFN) induction and/or response. The resulting chimeric oncolytic virus armed with the P gene exhibited reduced IFN sensitivity, diminished IFN induction capacity and enhanced oncolytic potency as compared to the control oncolytic MV (Haralambieva *et al.* 2007).

Modification of the viral vector for immune system evasion

In order to prevent immune rejection of the viral vectors, various strategies have been employed for their modification in addition to immunosuppression. One of the strategies involves deletion of the unnecessary viral genome sequences resulting in reduced viral protein expression. The reduced viral protein production results in less immune stimulation. This strategy has been successfully applied for reducing the immune response against the viral vector. For example, as described in another section, gutless Ad vectors devoid of most of the genome sequences have been reported to have improved transgene expression and enhanced safety profile (Morsy *et al.* 1998; Schiedner *et al.* 1998).

Another strategy for immune evasion is based upon serotype change of the viral vectors. Serotype specificity is one of the ways to classify subtypes of viruses. Per definition, antibodies generated against one viral serotype do not recognize another viral serotype. Based on this consideration, Riviere *et al.* demonstrated that different recombinant AAV serotypes, AAV type 1, 2 and 5, can be utilized for repeated cross-administration for transgene delivery (Riviere *et al.* 2006). This is because pre-existing immunity against one serotype of a viral vector does not prevent administration of another serotype of that viral vector. Another such example is provided by Ad vectors that express capsid proteins derived from two different serotypes, so called chimeric vectors. In this regard, it has been reported that the major antibody response is generated against the hexon capsid protein of Ad vectors. Based on this consideration, Wu *et al.* constructed a chimeric adeno-virus, Ad5/H3, by replacing the Ad5 hexon gene with the hexon

gene of Ad serotype 3 (**Fig. 3**). They demonstrated that antibodies against either the parent virus with the Ad5 hexon protein (Ad5/H5) or the chimeric virus with Ad3 hexon protein (Ad5/H3) did not cross-neutralize the other virus. In addition, pre-immunization of C57BL/6 mice with either of the viruses did not prevent subsequent infection by the other virus (Wu *et al.* 2002a). Thus, serotype switching strategies can be utilized for re-administration of the viral vectors. However, for each re-administration, a vector with different serotype will be required. Generation of these serotype viral vectors requires much effort and they may not transduce the same target cell population.

In addition to the above genetic modification strategies, viral vectors have also been modified through chemical strategies, most notably by the use of poly(ethylene glycol) (PEG) to mask the antigenic epitopes on the viral surface. This is also known as 'stealthing' (**Fig. 3**). PEG is a hydrophilic molecule, which physically masks the capsid proteins, thereby resulting in reduced innate immune response generated against the viral vector (Mok *et al.* 2005). Croyle *et al.* showed that PEGylated gutless Ad vectors could be re-administered with efficient transgene expression. Thus PEGylation can be utilized for improving the safety and efficacy profile of the viral vectors (Croyle *et al.* 2005). However, an immune response will still be generated against the new viral progeny produced in infected cells.

Recently, PEGylation-based immune evasion has been combined with molecules utilized for retargeting of the viral vectors to the desired cell types. For example, folate was chemically conjugated to PEG. The resulting folate-PEG was subsequently coupled to Ad vectors. This approach increased the transgene expression in folate receptor over-expressing cell line (KB cells) as compared to the folate receptor deficient cell line (A549 cells). In addition, PEGylation significantly reduced the innate immune response against the Ad vector (Oh *et al.* 2006). Thus, this combinatorial approach efficiently protects viral vectors from the innate immune system and also aids in efficient transgene delivery to specific target cells.

The examples listed above illustrate the various strategies that have been utilized for protecting the viral vector from the host immune system. However, the immune response generated against the viral vector and/or the delivered transgene can also be utilized in substituting immunity against the tumor cells. Although in general an immune response should be avoided to achieve a sufficient therapeutic effect, in the context of cancer immunotherapy this response is actually desired to efficiently utilize the capacity of the host immune system to kill the tumor cells. In this regard, viral vectors have been utilized for developing immunity against tumor-associated self antigens and thereby break tolerance. For example, AAV-2 was utilized to deliver BA46 to dendritic cells. BA46 is a membrane-associated glycoprotein that is expressed in most breast tumor cells, but not in general hematopoietic cell populations. The AAV-2 mediated BA46 delivery to dendritic cells resulted in generation of cytotoxic T lymphocytes against BA46 populations, which could potentially kill the breast cancer cells (Liu *et al.* 2005). Another example is provided by an Ad vector encoding HER2. The HER2/*neu* oncogene encodes for a protein p185 (C-erbB2). This protein is over-expressed in 30-50% of human breast cancer and in several other types of carcinomas. p185 has high oncogenic potential and its increased expression correlates with tumor aggressiveness. Ad-HER2 was injected intra-muscularly in BALB/c mice that are transgenic for the transforming form of the *neu* oncogene. These mice spontaneously develop carcinomas in all mammary glands. The Ad-HER2 vaccination resulted in both T and B cell responses against HER2, thereby preventing tumorigenesis (Gallo *et al.* 2005). Thus, viral vectors can potentially be utilized for generating immune response against the tumor cells.

The above examples highlight a few of the strategies that have been successfully used to counter the immune response that is generated upon viral vector administration such as immunosuppression, expression of immune suppression genes and genetic as well as chemical vector modifications. In addition, the immune response generated against the viral vector and its transgene has been exploited for developing patient's immunity against the tumor cells.

CELL-BASED STRATEGIES FOR CANCER GENE THERAPY

In addition to the virus-based strategies described above, viruses have also been utilized for cell-based strategies aimed at cancer gene therapy. Many of these strategies are centered on using cells as factories to produce angiogenesis inhibitors or cytokines that prime the immune system. Other strategies are aimed at using cells as "trojan horses" to deliver suicide genes or oncolytic viruses directly within the tumor stroma. Cell vehicles used as factories can result in the localized and sustained production of therapeutic proteins, the length of which depends on the type of vectors used for gene transfer, the cellular targets transduced, and the immunogenicity of the therapeutic proteins produced.

Therapeutic effector molecules for cell-based therapy

Angiogenesis inhibitors, such as angiostatin (O'Reilly *et al.* 1994) and endostatin (O'Reilly *et al.* 1997), are effective at limiting tumor growth and metastasis, but the fact that micrometastatic lesions can lay dormant may require continuous production to prevent future tumor outgrowth (Scappaticci 2002). Gene therapy approaches may be ideal for these situations, since these strategies allow for localized and sustained production, and avoids the need for the doses required for systemic efficacy (Persano *et al.* 2007). Mesenchymal stem cell mediated delivery of IL-12 was recently reported to reduce the formation of lung metastasis in a murine melanoma model, although NK and T cell mediated responses were also involved in the outcome (Elzaouk *et al.* 2006). A recent study by Jin *et al.* describes the combined use of an Ad vector that targets expression of an anti-angiogenic factor to the tumor endothelium along with a conditionally-replicating oncolytic Ad vector containing a tumor-specific promoter (Jin *et al.* 2005). A similar approach can be envisioned, using cell-mediated delivery of both therapeutic and oncolytic vectors. Combined therapeutic strategies for a disease marked by such vast epigenetic differences will likely be required. The true potential of angiogenesis inhibitors may be in the fact that they allow time for additional therapeutic avenues to take effect.

Cytokines are also favored as key therapeutic products for cell vehicle mediated delivery. As with angiogenesis inhibitors, large doses are often required to achieve therapeutically relevant concentrations. However, unlike angiogenesis inhibitors, elevated cytokine concentrations can have adverse effects (Lejeune *et al.* 1998; Neri *et al.* 2006). Thus, cellular vehicles may also serve to express and secrete the requisite cytokines for localized production at concentrations that limit untoward complications to the host. These cellular factories also abrogate the need for recombinant protein production and purification techniques. Minuzzo *et al.* recently provided a detailed review of the combined use of viral vectors with cell-mediated delivery of cytokines (Minuzzo *et al.* 2007).

Cancer gene therapy studies have also evaluated the use of prodrug activating enzymes, or suicide genes, that convert an exogenously provided substrate into a cytotoxic molecule. The herpes simplex virus thymidine kinase gene (HSV-TK) acts as a suicide gene in the presence of the guanosine analog, gancyclovir (GCV) (Elion 1980; Moolten 1986). Cell vehicles that express these suicide genes and engraft tumors can cause a 'bystander effect', or collateral damage to surrounding tumor cells upon addition of the prodrug (Freeman *et al.* 1993). Tumor cells, endothelial cells, progenitor cells, and mesothelial cells have all been evaluated as vehicles to deliver the HSV-TK/GCV medi-

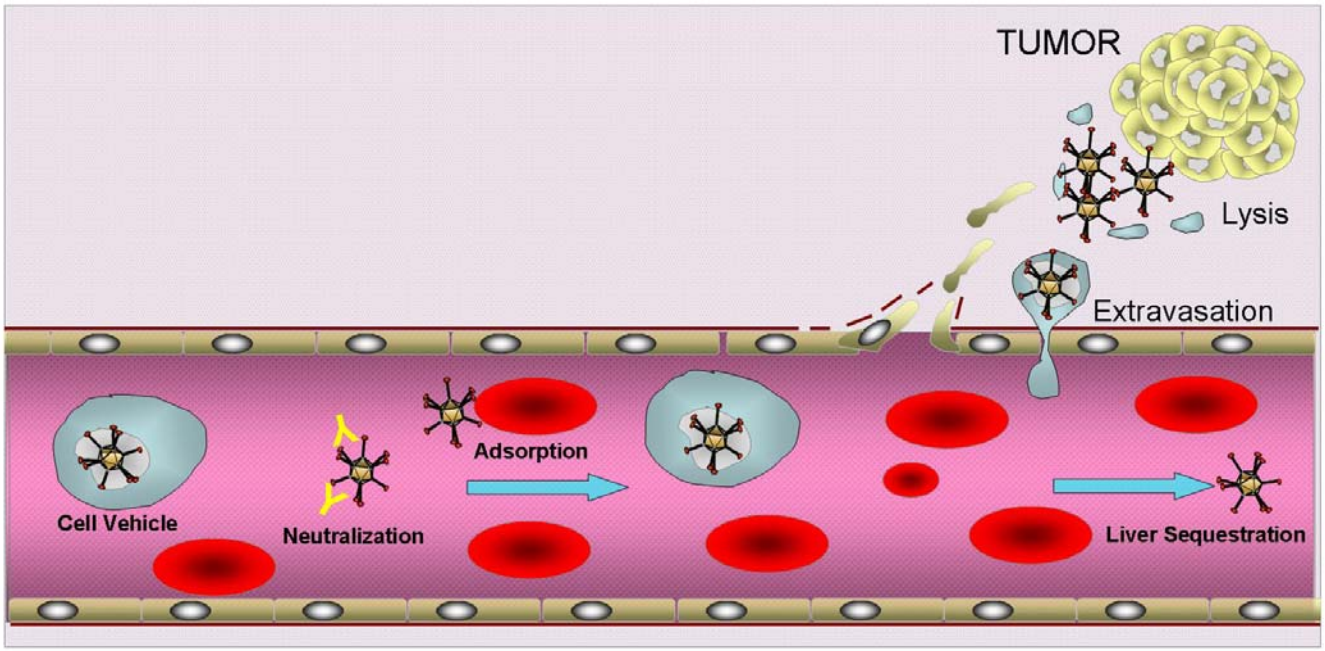

Fig. 4 Fate of systemically delivered Ad vectors. Systemically administered Ad vectors are not able to escape the circulatory system and are thus rapidly sequestered by cells of the reticuloendothelial system. Furthermore, Ad targeting is limited by soluble immune factors, such as complement and neutralizing antibodies, and non-specific interactions with erythrocytes, neutrophils, and monocytes. In contrast, cells that have intrinsic or engineered targeting activity can be loaded with Ad vectors and serve as site-specific delivery vehicles that protect virions from inactivation, while amplifying the payload in transit.

ated bystander effect to tumors (Rancourt *et al.* 1998; Coukos *et al.* 1999; Pereboeva *et al.* 2003; Rancourt *et al.* 2003).

Recent studies have centered on the use of cell vehicles to deliver oncolytic adenovirus vectors. This strategy avoids complications and the marked inefficiency associated with systemic introduction of viruses, such as preexisting neutralizing antibodies, non-specific vector sequestration in the liver or blood, and the inability to cross the endothelial barrier (**Fig. 4**) (Chirmule *et al.* 1999; Tsujinoue *et al.* 2001; Shayakhmetov *et al.* 2004; Franceschi 2005; Shayakhmetov *et al.* 2005). As described above, the list of naturally occurring, or recombinant oncolytic viruses includes adenovirus, herpes (Martuza *et al.* 1991), vaccinia, reovirus (Coffey *et al.* 1998), poliovirus, and Newcastle Disease Virus (Cassel *et al.* 1965; Martuza *et al*. 1991; Bischoff *et al*. 1996; Coffey *et al*. 1998; Timiryasova *et al.* 1999; Gromeier *et al.* 2000). Various cellular vehicles have also been employed to deliver these agents to tumors. Tumor cells infected with oncolytic parvovirus (Raykov *et al.* 2004) or Ad (Garcia-Castro *et al.* 2005) vectors have been shown to engraft and deliver the oncolytic payload to preexisting metastatic nodules. Others have used mesenchymal progenitors cells to deliver oncolytic agents to lung (Stoff-Khalili *et al.* 2007a) or intraperitoneal (Komarova *et al.* 2006) tumor xenografts. Cytokine induced killer cells have inherent tumor killing activity that is enhanced if the cells are preloaded with oncolytic vaccinia virus (Thorne *et al.* 2006). Iankov *et al.* recently reported the comparison of several cell vehicles as oncolytic measles virus carriers (Iankov *et al.* 2007). This strategy transferred the virus via a heterofusion mechanism, even in the presence of neutralizing antibodies, further demonstrating the true potential of this approach.

Cell types used in cell-based therapy

Along with the genetic payload to be used, the cell types suited or available for use as vehicles for cancer gene therapy will be critical. Different cell types have unique characteristics that may be required for efficient cancer gene therapy. In general, ideal cell vehicles are non-invasively accessible, can be purified and expanded to therapeutic levels, are susceptible to genetic manipulation, and home and engraft therapeutically-relevant target sites. Cell size is often a limiting factor due to the fact that systemic administration requires that the cells are capable of circulating through the lung microvasculature. Thus, the cells meeting most of the cell vehicle criteria are of hematopoietic origin, as these cell types are innately geared for systemic circulation. Further, many of the other characteristics defining ideal cell vehicles are natural properties of hematopoietic cells, including their ability to infiltrate tumor tissues.

Of the many leukocyte subsets found within the tumor stroma, tumor-associated macrophages (TAMs) are the most abundant, and are typically associated with poor prognosis (O'Sullivan *et al.* 1994; Leek *et al.* 1996; Takanami *et al.* 1999). Macrophages are essential components of innate immunity, acting as both antigen presenting and effector cells that protect the body against invading pathogens. Macrophages arise from progenitors in the bone marrow, entering circulation as promonocytes, where they differentiate into monocytes. Monocytes infiltrate tissues, further differentiating into resident macrophages. Macrophage infiltration and accumulation is a normal part of the inflammatory processes resulting from wounds and infection, as well as chronic inflammatory disease. Tumor cells secrete chemotactic molecules such as CCL2, macrophage-colony stimulating factor, and vascular endothelial growth factor that act to recruit TAM precursors. The tumor cells also secrete cytokines that polarize TAM into type II macrophages, which act to suppress adaptive immunity (reviewed by Mantovani *et al.* (2002)). Hypoxic conditions within tumors also induce expression of TAM genes associated with tumor cell proliferation, invasiveness, and angiogenesis (Murdoch *et al.* 2005). Although TAM are localized at the site of the tumor and play a part in tumor development, they lack the ability to home to tumors if isolated and systemically re-infused (Wiltrout *et al.* 1983; Ben-Efraim *et al.* 1994).

Many other leukocyte subsets are also found within the tumor stroma, including tumor-infiltrating lymphocytes (TILs). TILs have been shown to have either tumor-suppressing or tumor-promoting activity. $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs) suppress antitumoral immunity and thus promote tumor growth, while $CD8^+$ cytotoxic T

lymphocytes (CTLs) have direct tumor cell killing activity (Chen *et al.* 2005; Nishikawa *et al.* 2005). Unlike TAMs, TILs can be isolated, expanded *ex vivo*, and home to tumors when systemically reimplanted into the patient. This adoptive transfer approach has recently been shown to be an effective strategy for the treatment of melanoma. Interestingly, unmodified (Dudley *et al.* 2002) tumor-reactive T cells, and T cells engineered with viral vectors to be tumor reactive (Morgan *et al.* 2006) have both demonstrated effective tumor regression in melanoma patients.

Several other non-hematopoietic cell types have also been evaluated as cell vehicles for cancer therapy. Progenitor cells are widely used for this strategy. These cells are rapidly recruited to sites of injury where they differentiate into the cellular components required to repair the damaged tissue (Mackenzie *et al.* 2001). The architecture of a rapidly developing tumor closely resembles damaged tissue in that it is often disorganized, inflamed, and hypoxic (Haroon *et al.* 2000). Not surprisingly, mesenchymal and endothelial progenitor cells are recruited to the site of the tumor and can contribute to malignant growth (Studeny *et al.* 2004).

The specific cell types used will largely depend on the types of tumors being targeted and the types of therapeutics intended for delivery. Systemic injection of cells, unless specifically targeted to the lung, should be restricted to hematopoietic cell lineages that can circulate through the microvasculature. Locoregional, or intratumoral injection of cell vehicles may utilize additional cell types. In the rare circumstances in which natural tumor-homing T cells are attainable, delivery of lytic viruses may not be the best option, as these cells have inherent tumor-killing activity. As previously mentioned, many non-tumor cells contribute to tumor cell growth. Cell mediated delivery of agents that target elimination of Tregs or TAMs within the tumor may also prove to be therapeutically useful.

FUTURE PERSPECTIVES

The above mentioned examples highlight the crucial role viral vectors play in gene therapy applications. However, problems related to efficient delivery of the transgene to target cells, long-term transgene expression and immune responses against the viral vector and infected cells have prevented utilization of the full potential of viral vectors. As noted above, various strategies have been employed to enhance the transgene delivery and expression and reduce viral toxicity. In future, continued progress in these respects will further improve overall efficiency of the viral vector based gene therapy.

Cell based therapy has utilized the many advances in viral vector mediated gene expression technology for concentrated, but localized delivery of therapeutic products. Although the idea of cell-based delivery of therapeutics has been around for quite a while, practical application has been limiting. Realization that particular cell types have true homing potential has led to revitalized interest in this technology. Much of the transcriptional and targeting knowledge obtained for both viruses and cells can now be combined for multifaceted cancer treatment approaches.

One of the interesting aspects related to tumor therapy is that combination of gene therapy with radiotherapy (Rogulski *et al.* 2000) or chemotherapy (Khuri et al. 2000) has shown synergistic effects for tumor treatment. Thus, a combinatorial approach has been determined to be optimal for tumor treatment. Therefore, most likely in future viral vectors will be combined with both existing treatments for cancer, as well as new treatment opportunities offered by for example, nanotechnology. As an example, gold nanoparticles (AuNPs), can be used for hyperthermic tumor cell ablation using laser irradiation (O'Neal *et al.* 2004). Everts *et al.* have attached AuNPs to Ad vectors to deliver these nanoparticles specifically to tumor cells (Everts *et al.* 2006). This complex of Ad vectors with AuNPs can potentially be used for simultaneous tumor treatment with gene therapy and nanotechnology approaches. These viral vectors with coupled nanoparticles have been previously defined as viro-nano therapy agents (Saini *et al.* 2006).

In conclusion, viral vectors as well as genetically modified cells are important for cancer gene therapy. Technological advances will further increase the utility of viral vectors for efficient gene and cell therapy in future, and much progress can be expected in the coming years, now that major roadblocks have been identified and strategies to overcome these roadblocks have shown promise in pre-clinical models.

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