Chapter 1

Translational Cancer Research: Gene Therapy by Viral and Non-viral Vectors

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Adenovirus

Adenovirus is among the most used vectors for gene therapy and gene transfer, and about 23% of all vector-based clinical trials have been performed with it (www.wiley.com/legacy/wileychi/genmed/clinical/). Adenovirus was first isolated in 1953 from human adenoids [1]. To date, 55 different human serotypes, subdivided into seven subgroups (A–G), have been characterized [2,3].

Adenovirus is a nonenveloped double-stranded DNA virus surrounded by an icosahedral protein capsid (Table 1.1). The capsid comprises penton and hexon proteins with knobbed fibers protruding from the vertices of the capsid [4]. Soon after its entry into the target cell viral DNA reaches the nucleus where starts its replication. Early genes, mainly involved in DNA replication, are transcribed first [5], followed by late genes mainly coding for structural proteins [4].

Adenoviruses tend to be species-specific with regard to permissivity to replication. However, there may be some exceptions to this general rule. It has been reported that adenovirus serotype 5 subgroup C (usually referred as Ad5, the most used gene therapy vector) can replicate to some degree in cotton rats [6,7], New Zealand rabbits [8], and Syrian hamsters [9]. This feature of Ad5 has been very important for scientists around the world because it has allowed them to use these animal models to develop new therapies for disease.

Historically adenovirus has been the most used vector for gene therapy and gene-transfer purposes. In 1970s F. Graham and colleagues discovered the importance of the E1 gene, that made possible the use of adenovirus as a viral vector for gene therapy [10]. In fact, as E1 gene products initiate the replication of the viral DNA, serotype 5 adenoviruses with E1 deleted are incapable of replicating and remain episomal. Taking advantage of this characteristic, scientists replaced E1 with different expression cassettes to avoid virus replication while promoting expression of the transgene inserted in place of E1. Later on, E1-deleted adenoviral vectors, also known as first-generation adenoviral vectors (FG-Ad), were developed into high-capacity adenoviral vectors or Helper-dependent adenoviral vectors (Hd-Ad). HD-Ad are devoid of all viral genes except the two inverted terminal repeats (ITRs) and the packaging signal (psi). They show a high cloning capacity (up to 36 kb) and reduced immunogenicity and toxicity [11] (Figure 1.1). Since then, it has been mainly used as vector for gene transfer for genetic diseases [12] or to treat cancer [13]. The immunogenicity of adenovirus may render it unsuitable for long-term...
gene expression but makes it attractive for treatment of cancer. Use of a replication-deficient adenovirus as a gene delivery vehicle is the classic approach, with some exciting clinical results [14,15,16,17], but no products have been approved outside of China. This approach has been reviewed recently [18]. In the past decade, many adenoviral gene therapists have focused on use of adenovirus as a replication-competent oncolytic virus and thus this will be focus of this chapter.

### Oncolytic Adenoviruses for Treatment of Cancer

Oncolytic adenoviruses are specifically modified to selectively replicate in and destroy cancer cells. This selectivity is achieved by modifications of the genes involved in viral replication so that the life cycle of the virus can occur only in cells than can transcomplement the defect, including cancer cells, while the replication of the virus is arrested in normal cells (transcriptional targeting) (Figure 1.2). An alternative approach is to use tumor-specific promoters to “drive” E1 expression to allow selective replication of the virus in cancer cells [19] (Figure 1.2).

Historically, the first adenoviruses used in patients were wild-type viruses [20]. The concept was revived with the first adenovirus proposed to have tumor selectivity, dl1520 (today known as ONYX-015) [21]. This adenovirus bears a naturally occurring variation that results in a nonfunctional E1B-55k product. E1B-55k is one of the proteins encoded by the early gene E1 and its normal protein function is to mask host cell DNA. This virus is replication-deficient in normal cells but replicates in cancer cells.

### Table 1.1 The main characteristics of the viruses discussed in this chapter.

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Adenovirus</th>
<th>Vaccinia virus</th>
<th>Herpes simplex virus</th>
<th>Parvovirus</th>
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<tbody>
<tr>
<td>Genome</td>
<td>Linear dsDNA, 36 kb</td>
<td>Linear dsDNA, 200 kb</td>
<td>Linear dsDNA, 150 kb</td>
<td>Linear ssDNA, 5.1 kb</td>
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<td>Transgene capacity</td>
<td>≈3 kb</td>
<td>&gt; 25 kb</td>
<td>&gt;25 kb?</td>
<td>&lt;4.6 kb</td>
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<td>Replication-competent vectors</td>
<td>FG-Ad: 7.8 kb</td>
<td>MVA, ΔD4R: &gt; 50 kb</td>
<td>HSV amplicon: =150 kb</td>
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<tr>
<td>First-generation adenoviral vectors</td>
<td>HD-Ad: ≈36 kb</td>
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<td>Helper-dependent adenoviral vectors</td>
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<td>Genetic targeting</td>
<td>• Deletion of genes essential for replication in normal cells (E1A, E1B)</td>
<td>• Deletion of genes essential for replication in normal cells (VGF, TK)</td>
<td>• Deletion of genes essential for replication in normal cells (TK, RR, γ34.5)</td>
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<td>• MicroRNA targets in genome</td>
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<td>Particle retargeting</td>
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<td>• Cell-specific peptides in fiber/knob</td>
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dsDNA, double-stranded DNA; HSV, herpes simplex virus; MVA, modified vaccinia Ankara; ssDNA, single-stranded DNA; TK, thymidine kinase; VGF, vaccinia growth factor.
Figure 1.1 Schematic diagram representing the different kinds of adenovirus-derived vectors used for gene therapy. (A) Wild-type adenovirus is able to replicate and kill all permissive cells. (B) The E1 gene is replaced by the expression cassette; this vector can infect all permissive cells but they cannot replicate unless E1 is transcomplemented by the packaging cell line. (C) All viral genes are deleted except ITRs and the packaging signal. These vectors can infect all permissive cells but they cannot replicate. (D) Oncolytic adenoviruses. These viruses have been engineered to selectively replicate in and kill cancer cells.
function is to promote the degradation of p53 to avoid the infected cell undergoing apoptosis [22]. In infected normal cells p53 is not degraded by the mutated E1B-55k so that they can smoothly continue towards cell cycle arrest and apoptosis, which causes the arrest of the virus’s life cycle; on the other hand, in cancer cells, where the p53/p14ARF pathway is universally defective, the mutation is not needed to avoid apoptosis [21]. An issue with this type of virus is that E1B-55k is needed for late mRNA transport and its absence results in ineffective oncolysis, several orders of magnitude less than with the wild-type virus [23].

An alternative strategy used to generate adenoviruses selective for cancer cells is a 24bp deletion of the E1A gene [23,24,25]. This deletion results in the inability of E1A to bind to retinoblastoma tumor-suppressor protein (Rb) and to release
eukaryotic initiation factor E2F, which in the case of wild-type adenovirus would result in S-phase induction in normal cells. Therefore the “delta-24” viruses are unable to induce S-phase in host cells and no viral replication follows. In contrast to normal cells, most if not all cancer cells have a defective Rb/p16 pathway, rendering the Rb-binding property of E1A dispensable [26]. An important difference to dl1520 is that these types of viruses are not attenuated in comparison to wild-type adenovirus with regard to replication in cancer cells [24].

Another strategy to restrict virus replication to tumor tissue is to drive E1A gene expression with a tumor-specific promoter. The first example of this type of modification was an adenovirus with prostate-specific antigen promoter driving expression of E1A [27]. Since then a multitude of different tissue-specific promoters have been used, including α-fetoprotein for hepatic cancer [28], tyrosinase for melanoma [29], and carcinoembryonic antigen (CEA) for colorectal cancer [30]. Also, tissue-specific promoters that are activated in a variety of cancer types have been employed, including cyclo-oxygenase 2 promoter [31,32,33,34], L-plastin promoter [29,35], and human telomerase reverse transcriptase promoter [36,37]. The selectivity of dl1520 and delta-24 occurs after E1 expression, while tumor-specific promoters act prior to E1 expression. Therefore, an appealing approach is to combine both [33].

In addition to these strategies that restrict viral replication to tumor cells (transcriptional targeting), effort has also been put into modifying the adenovirus capsid to increase transduction of cancer cells (transductional targeting) (Figure 1.3). To this purpose different serotypes or chimeras have been tested to increase tumor transduction (recently reviewed by Cerullo and colleagues [13]). Particularly noteworthy has been the adenovirus 5/3 chimera. This modified adenovirus has been generated by placing the Ad3 fiber knob into the Ad5 backbone, resulting in an Ad5/3 chimera that displays the cell-binding properties of serotype 3 [38,39]. These chimeras also exhibit enhanced gene delivery and efficacy in preclinical animal models [39,40,41]. Recently this approach was taken a step further by developing the first fully serotype 3 (Ad3)-based oncolytic adenovirus, which has shown very encouraging results in animal models and human patients [42,43]. Transcriptional targeting is fully compatible with transductional targeting and an appealing concept is to combine them, as seen in many advanced-generation viruses [33].
Importantly, oncolytic adenoviruses have also been used as delivery vehicles to produce molecules (such as antibodies, drugs and prodrugs, cytokines and chemokines, and so on) directly at the tumor site [13]. This approach has been particularly helpful because it allows, especially for molecules that have high systemic toxicity, a high local concentration associated with less systemic exposure.

For this purpose, a common way to insert foreign DNA into the adenovirus genome is by replacement of small proteins encoded by early or late genes. Transgenes can completely replace $E3$ [44] or just part of this gene [26,28]. Transgenes can also be inserted in the late genes and the expression level of the transgene could depend on the insertion site [45].

A multitude of different proteins have been investigated as “arming devices” for oncolytic adenoviruses. Tumor-suppressor genes such as $p53$ have been used to enhance oncolytic cell killing regardless of the $p53$ status of the cancer cell line [46]. Prodrug-converting-enzyme-based systems commonly employ either cytosine deaminase for 5-fluorocytosine conversion to 5-fluorouracil [35,47], HSV-tk for ganciclovir conversion to its active metabolite [48], or both [49]. Antiangiogenic molecules have also been used for arming [50], in addition to various other molecules such as human sodium iodide symporter, which has been used to concentrate radioiodine in target cells [51]. Furthermore, immunostimulatory cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF) [52,53,54,55] used to boost antitumoral immunity have been under active investigation as transgenes.

Another interesting approach recently explored for adenovirus has been the enrichment of its genome with TLR9-specific sequences to increase TLR9 stimulation and consequently to enhance the antitumor immunity [56]. Along the same line arming the adenovirus with ligand for CD40 has also shown enhanced antitumor immunity in animal models and in cancer patients as well [57,58]. Unfortunately oncolytic viruses – which need most of their genome to replicate – have a limited cargo size capacity. A larger payload can be achieved by FG-Ad or HD-Ad but these vectors are not capable of replicating in cancer cells. In fact it would be interesting to combine the cloning capacity of HD-Ad with the killing capacity of oncolytic viruses; these approaches are under investigation. This should be feasible as oncolytic viruses have already been utilized to amplify first-generation vectors to combine the merits of each [59].

**Use of Adenovirus in the Clinic**

The observation that wild-type adenoviruses can kill cancer cells has been acknowledged for a long time. In fact, the first in-human use of adenovirus was in the 1950s [20], when 10 different serotypes were used to treat 30 cervical cancer patients. The treatments were quite safe, which is remarkable considering that wild-type viruses were used. With regard to efficacy, two-thirds of the patients had a “marked to moderate local tumor response” [20] with necrosis and ulceration of the tumor. Although “response” was not defined, these numbers are not so far from what is seen with modern viruses [58,60].

Since then, a multitude of different oncolytic adenoviruses have been conceived and tested in human clinical trials for different tumor types such as pancreatic cancer [61], brain tumors [62], prostate cancer [63], bladder cancer [64], and ovarian cancer [65], among others.

The first oncolytic adenovirus used in clinical trials in modern times was ONYX-015. More than 300 cancer patients with different tumor types were treated in several clinical trials from phase I to phase II, but a phase III trial was never initiated in the West. Instead, in China a similar virus, H101, was rapidly taken through all phases and approved in 2006 as Oncorine [66]. The overall results from these clinical trials were that this virus is safe and selective for cancer [67], and has antitumor efficacy, especially when combined with chemotherapy. However, preclinical data suggest that the oncolytic potency is up to 100 times lower than, for example delta-24-type viruses [25]. Also, an unarmed virus might be at a disadvantage compared to armed viruses.

Recently, scientists have realized that the use of oncolytic adenoviruses for treatment of cancer is particularly intriguing given their ability to wake up the immune system, stimulating a response to the cancer [13,68]. Although a clear mechanism on how this happens still remains to be fully clarified, we believe that even in the
tumor-immunosuppressive microenvironment adenviral particles have the ability to (i) stimulate dendritic cells, predisposing them to cross-priming, (ii) promote antitumor immunity by enhancing the release of tumor-associated antigens in the presence of a danger signal [69], and (iii) break tolerance of the immunosuppressive tumor environment through interaction with pathogen-associated molecular pattern receptors [11,70,71,72,73]. These “natural” features can be further improved when adenviruses are armed with immunostimulatory molecules.

We have been among the first laboratories to demonstrate the involvement of the immune system in human cancer patients. In our first study patients were treated with a GM-CSF-encoding serotype 5 virus (Ad5D24-GM-CSF) bearing a 24 bp deletion in E1A [68]. We assessed the tumor-specific immune response by ELISPOT and by flow cytometry. ELISPOT was performed on fresh peripheral blood mononuclear cells pulsed for 12 h with tumor-specific and adenvirus-specific pools of peptides. Tumor specificity was assessed using survivin as an example of a pan-carcinoma antigen commonly expressed by most tumors [74].

Similar immunological data were observed with a serotype chimera 5/3 (Ad5/3D24-GM-CSF) [60] and with an integrin-targeted virus [75]. In an interesting contrast, when an unarmed oncolytic adenvirus (Ad3hTERT) was used in humans, antiviral responses were equally emphatic but less evidence of antitumor response was seen [42]. It remains to be studied how important the immunostimulatory transgene is or if the serotype also plays a role.

Interesting results have also been reported by Li et al. [76]. They present the data of a phase I dose-escalating trial with an oncolytic adenvirus expressing the heat shock protein 70 (HSP70), emphasizing some aspects of the antitumor immune-mediated response. Specifically they observed elevation of the number of CD4+ and CD8+ T cells as well as natural killer (NK) cells in the blood of the patients after the administration of the virus [76].

Similar results were also reported in another phase I trial with an oncolytic adenvirus expressing GM-CSF [54]. Similarly an important involvement of the immune system was also demonstrated with CD40L-expressing oncolytic adenviruses [57,58].

**Vaccinia Virus**

Vaccinia virus (VV) is best known for its use as an efficient vaccine against smallpox, which led to the worldwide eradication of the disease. Due to this important historical role, VV has the longest and most extensive history of use in humans of any virus and a wealth of basic, preclinical, and clinical data are now available. Different strains of VV exist, of which modified vaccinia Ankara (MVA), New York VV (NYVAC), Lister, Wyeth, and Western Reserve (WR) are the most commonly used. Some of these (MVA, NYVAC, Lister) are completely or partially replication-deficient and are therefore mostly used as gene-transfer vehicles and vaccines. Wyeth and WR instead are mostly employed as replicating viruses in experimental cancer therapies due to their strong oncolytic properties. VV has an approximately 200 kb double-stranded DNA genome, which encodes about 200 genes (Table 1.1). The large, enveloped, and brick-shaped virus particles are about 300 nm in the longest dimension. VV does not require specific cell-surface receptors for transduction of target cells. Rather, it enters cells through membrane fusion or macropinocytosis. Thus, VV is able to infect a wide range of cell types. Upon entering the cytosol, VV immediately starts transcribing a defined set of early mRNAs using the transcription enzymes that the virus brings with it. Subsequently ribosomes and other components of the host cell translation machinery are recruited into defined granular structures called virus factories. Here viral gene replication and viral protein production take place and new infectious viral particles are formed. The majority of the new particles are intracellular mature virions, which have a single lipid bilayer envelope and remain inside the cell until lysis. However, a small subset of the viral particles wrap themselves in an additional lipid bilayer derived from the trans-Golgi network before egressing from the cell as extracellular enveloped virions (EEVs). The EEV particles can spread efficiently through the system of the host and are largely shielded from the immune system; therefore, they are also known as stealth particles.
**VV for Treatment of Cancer**

VV has several unique features that make it an attractive cancer gene therapy vector. Firstly, VV has a wide host range and is able to efficiently transduce a broad range of mammalian cell types *in vitro*. However, *in vivo* after intravenous injection in mice VV exhibits a natural tropism to tumors, which has been suggested to be due to the leaky vasculature in cancer tissue that allows the large virus particles to enter. Another advantage is that VV can hold up to 25 kb of foreign DNA, allowing the insertion of large genes and/or multiple expression cassettes. Furthermore, for efficient expression of the genes of interest, a number of strong viral promoters exist. Another advantage is that VV is a highly immunogenic agent that triggers a strong antibody and T-cell response, making VV an efficient vaccine vector. Moreover, replication-competent VV strains are highly oncolytic due to their rapid and efficient replication cycle, resulting in strong antitumor efficacy. Lastly, VV does not enter the nucleus at any time during the infection; thus, there is no danger of insertional mutagenesis. These attributes make recombinant VVs attractive agents for the treatment of many diseases, especially cancer. For cancer therapy VV has mainly been used in three ways: (i) as a replication-deficient expression vector of therapeutic genes; (ii) as a replication-competent, oncolytic virus; and (iii) as a vaccine expressing cancer epitopes and/or immune-stimulatory molecules. Many VV constructs, some of which combine the above-mentioned mechanisms of action, have been generated and evaluated preclinically and some of them have entered clinical trials with exciting results.

A Lister strain VV that expresses p53 is one example of the use of VV as a gene-transfer vector. This vector showed antitumor efficacy and minimal toxicity in a murine glioma model, even when irradiated with ultraviolet light to make it completely replication-incompetent [77]. However, despite the high transduction efficiency of VV, penetration within the tumor and infection of all cancer cells is generally difficult to achieve due to intratumoral barriers. Consequently, vectors expressing therapeutic genes with a bystander effect, such as an MVA strain VV expressing FCU1 (a secreted suicide gene) that has been evaluated in colon cancer models, have shown promising results [78]. Another strategy to improve tumor penetration and increase transgene expression inside the tumor is to use replication-competent VVs that at the same time kill cancer cells by oncolysis. There are many examples of oncolytic VVs – mostly of the Wyeth and WR strains – that have shown promising antitumor efficacy in various preclinical cancer models. To ensure tumor-selective replication, viral genes that are necessary for replication in normal cells but dispensable in cancer cells can be deleted. In line with this strategy, disruption of the viral thymidine kinase (TK) and vaccinia growth factor genes, which are both complemented in cancer cells but not in normal cells, significantly increased tumor specificity [79]. To enhance antitumor efficacy of oncolytic VVs, therapeutic genes can be incorporated into the vectors. Examples include VVs armed with suicide genes, antiangiogenic molecules, and immune-stimulatory proteins (reviewed in [80]). Vectors expressing immune-stimulatory agents might be particularly effective since induction of antitumor immunity could be the key to successful and sustained therapy. JX-594, a Wyeth strain VV deleted in TK and expressing GM-CSF, is an example of an oncolytic VV with enhanced immune-stimulatory properties that has demonstrated convincing preclinical results and is currently being evaluated in phase II clinical trials with encouraging preliminary results (reviewed in [80]). In most clinical studies so far, VV has been injected intratumorally, which is thought to be the safer route of administration. However, animal models and a clinical trial have shown that intravenously administered Wyeth and WR viruses have the potential to reach the tumor with minimal effect on normal tissue [81]. This opens up the possibility of reaching multiple tumor sites and metastases with a single virus injection, which would be highly desirable.

**VV as a Cancer Vaccine**

For cancer immunotherapy approaches, VV can be used to deliver nonspecific immune-modulatory molecules or tumor-specific antigens. Examples of immune-stimulatory molecules that have been delivered by VV include CD40L, GM-CSF, interleukin (IL)-2, B7-1, intracellular
cell-adhesion molecule (ICAM)-1, and lymphocyte function-associated antigen 3 (LFA-3 or CD58). These factors are cytokines and costimulatory molecules that are expected to alter the local immune-privileged tumor environment, which could lead to the host’s immune system attacking the primary tumor site as well as distant sites and metastases. An MVA vector expressing the combination of B7-1, ICAM-1, and LFA-3 (designated TRICOM) has been particularly efficient in activating T cells, which are among the most efficient immune cells in cancer therapy [82]. VVs expressing tumor-associated antigens are another attractive approach in cancer immunotherapy. The rationale is that the host’s immune system would be primed for antigens that are present on tumor cells but absent in normal tissue. Many different antigens have been incorporated in VVs, such as CEA, prostate-specific antigen (PSA), and the oncofetal antigen 5T4. VVs expressing these antigens showed significant antitumor efficacy in various animal models primarily due to induction of cytotoxic T cells. In clinical trials VV-CEA and VV-PSA have demonstrated antitumor efficacy especially when used in a prime-boost scheme in combination with avipox vectors. Trovax, a VV expressing 5T4, has been evaluated in phase II clinical trials in patients with metastatic colorectal cancer, metastatic renal cancer, and prostate cancer demonstrating strong 5T4 immune responses, which correlated with indicators of clinical benefit [83]. A phase IIb trial with an MVA expressing the tumor-associated antigen MUC1 and IL-2 (TG4010) in lung cancer in combination with chemotherapy as a first-line therapy suggested that the therapeutic vaccine improves clinical outcome [84]. Toxicity of VVs as cancer vaccines has generally been low in clinical trials, mostly consisting of transient fever, malaise, skin reactions, and pain at injection site.

In summary, VV seems to be well suited as a gene-therapy agent in cancer therapy in particular because of its high transduction efficiency, strong oncolytic effect, and immune-stimulatory properties. Based on the good safety profile of VV, the field will likely move towards developing more efficient cancer vaccines, combining immune-stimulatory features with oncolytic potential.

**Herpes Virus**

**General Properties and Life Cycle**

Herpes simplex viruses (HSV) are double-stranded DNA viruses with a relatively large genome of about 150 kb encoding 100–200 genes (Table 1.1). The genome is packaged within an icosahedral protein capsid, which itself is surrounded by a host cell-derived double lipid membrane envelope. Holding the envelope in place is a layer of virus proteins called the tegument. The HSV genome consists of two sequences, UL and US, each of which is surrounded by a pair of inverted repeat sequences (IRL and IRS) [85]. A substantial portion of the genome consists of accessory genes, which may facilitate but are not necessary for completion of the virus life cycle in permissive cells. Thus, it has been estimated that replacement of up to 50 kb of accessory genes should be possible in HSV vectors without fully compromising the capacity of the virus to replicate.

HSV bears several virus-specific glycoproteins on its membrane, which mediate entry into cells through common cell-surface glycosaminoglycans, including nectin and heparan sulfate proteoglycans, giving the virus broad tropism. Infection starts by virion attachment to the cell-surface plasma membrane, followed by membrane fusion and release of the capsid into the cytoplasm. The capsid, in turn, is transported via microtubules to the nucleus, where it is disassembled and the genome is released into the nucleus through the nuclear pores. Virus replication ensues, beginning by transcription of early genes and culminating with either establishment of latency or release of new viruses by budding at the plasma membrane and, in many cases, death of the infected cell.

**Cancer Targeting by Recombinant Herpes Viruses**

Several members of the herpesviridae family have been found suitable for gene delivery and/or cancer targeting. The first genetically engineered herpes virus vectors were designed as vaccines against herpes and incorporated a deletion of the immediate-early gene ICP4. However, since ICP4 is an essential gene, the resultant recombinant vector is replication-defective [85]. Since then, these and other replication-defective vectors, such as HSV
amplicons, in which all virus DNA save for the inverted terminal repeats has been replaced with foreign DNA, have been used for cancer targeting in preclinical settings. As another example, so-called disabled infectious single-cycle HSV (DISC HSV) vectors were shown to mediate tumor regression when engineered to express GM-CSF [86]. While such vectors are useful for gene transfer and cancer gene therapy when armed with, for example, immune-stimulating cytokines, in cancer targeting replication-competent or replication-selective vectors have been favored.

The first replication-competent HSV vector, dl5ptk, featured a deletion of the UL23 gene encoding TK. In a landmark study which pioneered cancer targeting by any recombinant virus (not just in the context of HSV), Martuza and colleagues used TK-deleted HSV in nude mice harboring human glioma xenografts to substantiate the concept of killing tumor cells in living hosts while sparing normal healthy tissue [87]. However, TK-deleted herpes viruses were considered unfavorable for clinical application as they were neurotoxic at high doses and lacked sensitivity to common antivirals (a failsafe against unwanted virus replication). Instead, new constructs were engineered. For example, UL39, which encodes herpes virus protein ICP6, the ribonucleotide reductase, was deleted in hrR3, based on HSV type 1 strain 1716. This agent demonstrated similar safety and efficacy in glioma targeting in experimental models as the TK-deleted vector dl5ptk [88] and continues to be a popular vector for cancer targeting today.

Around the same time, another HSV gene was deleted, RL1, which encodes protein ICP34.5 (more commonly known as γ34.5). γ34.5 counteracts the antiviral effects of double-stranded RNA-activated protein kinase R (PKR) by promoting dephosphorylation of eukaryotic initiation factor 2α, thereby ensuring that host cell (and virus) translation is not shut down during infection [89]. In cancer cells, PKR is typically rendered inactive or its antiviral effects are stunted, which allows even RL1-deleted HSV to replicate in these cells but not in normal cells. The deletion markedly attenuates herpes virus pathogenicity by rendering the virus unable to replicate productively in neurons. A recombinant vector based on HSV strain 1716 (trade name SEPREHVIR) which lacks both copies of the RL1 gene, is being developed by Virttu Biologics (formerly Crusade Laboratories). This virus has shown excellent safety in both glioma and melanoma patients (47 and five patients, respectively; www.virttu.com). While the survival of one patient with glioma has given cause to continue clinical development of HSV1716 in glioma [90], no activity (e.g. necrosis or virus replication) was seen in biopsies of 20 patients with oral squamous cell carcinoma following three direct intratumoral injections of up to $5 \times 10^5$ plaque-forming units (PFUs) of HSV-1716 [91]. Importantly, both UL39- and RL1-deleted HSV vectors still carry the TK gene, which allows additional control of these viruses by TK-targeted antiviral drugs, such as ganciclovir.

### Multimutated HSV Recombinants in Clinical Use

Because of lingering safety concerns with single-gene-deleted recombinants, two or more genetic deletions have been tested in the same virus. These second- (multimutated) and third- (multimutated + armed) generation viruses have since become the mainstay in cancer gene therapy. The first HSV-1-based recombinant to enter clinical testing was G207, which is deleted for both copies of the RL1 gene and which has an insertion of LacZ in one of the UL39 genes [92]. This virus has so far undergone two phase I/Ib studies in glioblastoma and demonstrated good safety and tolerability as well as signs of virus replication by reverse transcriptase-mediated PCR in tumor tissue in the brain [93].

Another virus in clinical development, NV1020, is based on a recombinant virus R7020 originally designed as a vaccine for HSV-2 [94]. It contains several genetic modifications, such as TK under the control of the ICP4 promoter as well as glycoprotein sequences from HSV-2, meant to immunize hosts against this member of the virus family without altering replicative or pathogenic properties of NV1020 compared to parental HSV-1 [95]. This virus is being tested in patients with colorectal cancer metastases in the liver following intrahepatic arterial infusion. In a phase I/II trial, 50% of patients displayed stable disease with a
median time to progression of 6.4 months at the maximum tolerated dose ($1 \times 10^8$ PFU [96]). A follow-up phase II/III study is planned. The first third-generation recombinant HSV vector G47Δ features deletion of HSV gene $\alpha 47$ in addition to $RL1$ and $UL39$. In cells infected with wild-type HSV, $\alpha 47$ eliminates MHC I from the cell surface, thereby reducing the capacity of the cell to present antigen to the immune system and allowing the infection to go unnoticed. Deletion of this gene thus increases immunogenicity of the infection, and while it increases recognition of the virus by the immune system, it also facilitates bystander immune responses against the tumor, resulting in increased overall antitumor efficacy. HSV G47Δ demonstrated excellent antitumor efficacy in several preclinical models and a phase I safety study was initiated in 2009, with results still pending publication [97].

In addition to selectively engineered gene-deletion recombinants, HF10 is a laboratory clone of a stock HSV-1 virus which displays several gene deletions and other mutations compared to the oncolytic viruses described above. While its exact mechanisms of attenuation remain unclear, HF10 has displayed excellent antitumor efficacy in several different preclinical cancer models and has now undergone at least five phase I studies in cancer patients. Lastly, Oncovex-GM-CSF, currently known as talimogene laherparepvec or T-VEC, proved safe and efficacious in phase I/II studies in advanced melanoma (28% objective response rate) and has recently completed a phase III trial, the potentially groundbreaking results of which are anticipated in the near future. The virus, originally developed by Biovex and now being developed by Amgen, is based on HSV-1 and features deletions in $RL1$ and $\alpha 47$ genes in addition to expressing the cytokine GM-CSF, which may increase overall therapeutic efficacy of the vector by stimulating monocyte maturation [98]. However, the discovery that high intratumoral GM-CSF expression in some cancers is associated with heavy immune-suppressor cell infiltration (including myeloid-derived suppressor cells, tumor-associated macrophages, and neutrophils) would certainly warrant closer study of GM-CSF when expressed by recombinant viruses [98].

Other Herpes Virus Family Members with Cancer-Targeting Potential

The main advantage of herpesviral vectors is their capacity to carry large transgenes, exceeding 150kb in amplicons and up to 50kb in replication-competent vectors, while the main drawbacks include difficult cloning (even using new BAC systems), safety issues such as neurotoxicity at high virus doses, and the risk of recombination with or without activation of endogenous herpes viruses. In order to address some of these issues, other members of the herpes virus family have been tested as gene-delivery/-therapy vehicles. In addition to the vectors described above, several new laboratory strains of HSV-1 have been generated by serial passages on human cancer cells in cell culture [99]. These recombinants typically harbor multiple mutations throughout the genome that increase replication fitness in cancer cells while reducing it in normal cells. However, the safety and potentially new molecular mechanisms of such mutants warrants characterization to avoid unexpected side effects. In addition, herpes viruses are, like most viruses, prone to Muller’s ratchet and will lose fitness upon serial passages in a limited host cell repertoire, potentially reducing the usefulness of the passaged mutant viruses as broad-spectrum therapeutics [100].

Since HSV-2 is also a human pathogen with similar features to HSV-1, it has been tested for oncolytic potential. A vector called FusOn-H2 was created by deletion of the ribonucleotide reductase gene $ICP10$ (corresponding to the $ICP6$ gene in HSV-1), which abrogates the oncogenic potential of the vector and confers syncytium-forming properties [101]. FusOn-H2 was more efficacious in mouse tumor models than common vectors based on HSV-1. Other human herpes viruses developed for cancer targeting include Epstein–Barr virus (EBV) and human herpes viruses 6 and 7, which are noncytolytic but still able to efficiently transduce specific types tumor cells. EBV is a gamma-herpesvirus which displays an inherent tropism to lymphoid cells, particularly of the B-cell lineage, which is contrast to the neurotropic simplex subfamily members on which most oncolytic viruses are built. Therefore, EBV-based vectors could potentially carry less risk of neurological complications than vectors based on HSV-1, or,
correspondingly, EBV-based vectors may be better suited to target B-cell-related cancers than HSV-1 viruses. In the case of EBV, three genes have been removed from the virus to abrogate its capacity to transform cells, also rendering it replication-defective [102]. An attenuated vaccine strain of varicella zoster virus, causative of chicken pox in children and herpes zoster in adults, also demonstrated capacity to infect and kill human glioblastoma cells \textit{in vitro} [103]. Because the vaccine virus is safe for humans it may be possible to develop it for targeting brain (and other) tumors in the future.

As for nonhuman herpes virus family members, vectors based on equine herpes virus 1 were recently shown to possess oncolytic potential against human glioblastoma [104]. Also, both bovine herpes viruses 1 and 4 are being developed as oncolytics [105,106], with the latter demonstrating oncolytic potential in glioblastoma in mice. Further, vectors based on pig pseudorabies and monkey saimiri virus (prototype \(\gamma\)-2 herpes virus) show targeting potential in human cancer cells, although these viruses are generally not cytolysic on their own and require additional modification (i.e. "arming") to be useful as therapeutics. In addition, while, for example, saimiri virus can transduce human T cells, lingering concern about oncogenic potential may limit translation of these nonhuman herpes viruses into the clinic [107]. This would likely be a concern for vectors based on EBV and other potentially oncogenic viruses as well.

**Herpes Virus Retargeting**

Several different strategies have been developed to increase virus specificity. For example, a single-chain antibody-binding domain was successfully incorporated into a variable loop of glycoprotein G, allowing for coating of the virion by a single-chain antibody of choice. By this approach, the natural broad cellular tropism of HSV could be diminished and the virus retargeted to cells expressing antibody target receptors, such as human epidermal growth factor receptor 2 (HER-2). Such HER-2-targeted HSV vectors showed robust antitumor activity in a target-dependent fashion [108]. A more straightforward approach has been to restrict virus gene expression to desired cell types by placing the genes under control of cell-specific promoters. For instance, as an alternative to gene deletion, which reduces virus replication even in cancer cells, the main neurovirulence gene \textit{RL1} (encoding \(\gamma34.5\)) was placed under control of tumor-restricted promoters, which allowed the virus to replicate with nearly undiminished efficacy in several types of cancer cells [109]. Indeed, several other HSV genes, essential or not, have been tested under a variety of tissue-specific promoters, demonstrating the feasibility of directing HSV replication to only desired cell types/tissues and in some cases, due to relative promoter strengths, even increasing the replicative efficacy of the tissue-specific vectors compared to gene-deleted viruses [110]. Finally, HSV replication may be regulated through manipulation of virus gene transcripts by microRNA targets through which the cellular RNA-silencing mechanism may be harnessed to reduce translation of the targeted genes. It is possible to combine more than one targeting approach to maximize tissue specificity, as exemplified by a liver-targeted oncolytic HSV recombinant in which virus replication was on one hand restricted to the liver by a liver-specific promoter driving the essential virus glycoprotein gene H and on the other hand abrogated in normal liver cells through microRNA targets in gene H which are sensitive to RNA silencing in normal liver cells but not liver cancer cells [111].

**Parvovirus**

Parvoviruses (PVs) are small (from Latin \textit{parvus} meaning small; about 25 nm in diameter) icosahedral particles containing a single-stranded DNA of about 5000 nucleotides [112] (Table 1.1). Upon infection of permissive cells PVs undergo viral replication and release of virus progenies. Infection by some but not all PVs results in cell death. In order for the cell lysis to occur, the cell has to be of the right animal species, it has to be proliferating, and it has to be rather undifferentiated [113]. Many of these characteristics can be found in tumor cells, and in fact some PVs can be used as oncolytic viruses, in which case we will talk of oncolysis. On the other hand, other PVs are better suited for gene delivery, as they do not kill the infected cells. Most PVs are not known to cause any diseases in humans and thus they are often called "viruses in
search of diseases.” Some PVs such as adenoassociated virus are low in pathogenicity and immunogenicity and are popular vehicles for treatment of many types of genetic and metabolic diseases [112].

The most used and studied PV for treatment of cancer is the rat PV, called H-1PV, which is attractive for its capability of infecting and replicating in humans (including human cancer cells) without causing significant clinical signs due to death of normal cells [112].

The oncotropism of PVs is not due to better virus uptake by transformed cells, but to a more efficient replication in these cells. Specifically, the conversion of the viral single-stranded DNA to double-stranded replication forms, and the transcription of these duplex forms, depend on factors that are often dysregulated in cancer cells [cyclin A, E2F, and cAMP-response-element-binding/activating transcription factors (CREB/ATF) among others], allowing the virus to preferentially replicate (and kill) these cells rather than normal cells. The killing of cancer cells is mainly due to the accumulation of a cytotoxic protein called NS1. Interestingly, it has been discovered that not only are cancer cells more susceptible to NS1 but also that in these cells the concentration of this protein is often significantly higher than in normal cells; the mechanism for this is still unknown and under investigation. These characteristics make PV a selective anticancer oncolytic virus [112].

Given its efficacy and selectivity for tumor tissue, H-1PV has been particularly useful for treatment of brain tumors and non-Hodgkin lymphoma. Together with its oncolytic activity it has also been recently observed that PVs are able to influence the immune system to recognize the tumor [114].

As with other oncolytic viruses, the possibility of arming these platforms to generate more potent and more immunogenic devices makes them even more appealing for treatment of cancer [115]. Like adenovirus, PVs have also been enriched with TLR9-specific sequences to enhance antitumor immunity [116].

Recently a phase I/IIa trial has started in Germany for patients with recurrent glioblastoma multiforme (www.clinicaltrials.gov, identifier NCT01301430). This will be the first clinical trial with H-1PV in Germany.

**Final Remarks**

In conclusion, while gene delivery with nonreplicating viruses may have its uses in the treatment of cancer, replication-competent oncolytic viruses have become popular in the last decade. Although several RNA and DNA viruses are being studied, the latter are furthest along in clinical use due to their stability, predictability, ease of construction, and production. Predictable pharmaceutical properties are key regulatory aspects of clinical translation of “advanced therapy medicinal products.” The most popular oncolytic DNA viruses are adenoviruses, VVs, and herpes simplex viruses, all of which have entered randomized clinical trials; the first product approvals are expected by 2015. One virus, Oncorine, has already been approved in China. These viruses feature different characteristics and all of them are active in many different tumor types. Although a “magic bullet” to eradicate cancer as global disease will never be identified, it seems likely that viruses will enter the oncologist’s arsenal to design tumor-specific and patient-specific therapies. Immunotherapy is a missing sector in the pie chart of antitumor approaches and oncolytic viruses could contribute to filling the void. For this to be achieved it is of utmost importance to profoundly understand the biology of oncolytic viruses at the interface between the virus and host so that we might rationally design combination therapies that can attack tumors from different angles.

**References**


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