Replication-selective oncolytic viruses in the treatment of cancer

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In the search for novel strategies, oncolytic virotherapy has recently emerged as a viable approach to specifically kill tumor cells. Unlike conventional gene therapy, it uses replication competent viruses that are able to spread through tumor tissue by virtue of viral replication and concomitant cell lysis. Recent advances in molecular biology have allowed the design of several genetically modified viruses, such as adenovirus and herpes simplex virus that specifically replicate in, and kill tumor cells. On the other hand, viruses with intrinsic oncolytic capacity are also being evaluated for therapeutic purposes. In this review, an overview is given of the general mechanisms and genetic modifications by which these viruses achieve tumor cell-specific replication and antitumor efficacy. However, although generally the oncolytic efficacy of these approaches has been demonstrated in preclinical studies the therapeutic efficacy in clinical trials is still not optimal. Therefore, strategies are evaluated that could further enhance the oncolytic potential of conditionally replicating viruses. In this respect, the use of tumor-selective viruses in conjunction with other standard therapies seems most promising. However, still several hurdles regarding clinical limitations and safety issues should be overcome before this mode of therapy can become of clinical relevance.

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Cancer has become one of the leading causes of death in the Western society. As current cancer therapies, like radiotherapy and chemotherapy, have often only a limited effect, a strong need exists for novel approaches to the treatment of cancer. One of these approaches is the use of viruses.

Gene therapy seeks to treat cancer by the introduction of genes that will either kill cells by sensitizing them to other therapies, or by enhancing an immune response against transfected cells. Most of gene therapy strategies to date utilize replication defective viruses, because of the risk of uncontrolled viral spread when replication competent virus are given to patients. If, however, one's goal is to eliminate the transfected cells, as is the case in cancer, then one may take advantage of the inherent cytotoxic characteristics viral replication exhibits. The heavy biochemical demands that the virus makes upon its host cell in order to replicate, will often lead to apoptotic cell death.¹ In addition, upon completion of the virus replication cycle, many viruses induce lysis of the host cell, allowing further spread of the newly synthesized virus particles to other cells.² Since some viruses can directly damage and kill cells, one may exploit these pathogens to mediate cell lysis of specific cell populations, like tumors.

This idea of using replication competent viruses in the battle against cancer instead of using replication defective ones, has led to the rise of a novel mode of cancer therapy: oncolytic virotherapy.

In the beginning of this century, it was noticed that a patient with cervical carcinoma experienced significant tumor regression after rabies vaccination.³ In addition, there are reports of remissions of Burkitt's and Hodgkin's lymphomas following natural infection with measles virus.^{4,5} In the 1950s, human trials with several potentially oncolytic viruses were initiated.^{6–11} Although tumor regression was reported in some cases, these studies generally demonstrated lack of significant antitumor efficacy of this mode of therapy. As a result, this mode of therapy was abandoned for almost two decades. Advances in our understanding of the basic principles of viral biology, including cell cycle control and carcinogenesis, and the discovery of recombinant DNA technology in the intervening years have resulted in the current revival of oncolytic viruses as a cancer therapy. Following the first description of a virus engineered to replicate selectively in dividing cells a decade ago,¹² the field of oncolytic virotherapy has significantly expanded. Results from preclinical studies with several virus types have been quite promising, and several different virus species have entered or even completed clinical trials (Table 6).

Currently, a wide variety of virus types is under study that can be roughly divided into two groups. On the one hand, there are inherently oncolytic wild-type strains. On the other hand, several virus types are rendered

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tumor-selective by genetic modifications. This paper will focus on the biological principles underlying tumor-selective replication of these viruses and strategies by which their oncolytic potency can be improved, with particular emphasis on the use of these viruses in multimodal antitumor regimens. In addition, an outline will be given of the current status of the field concerning viruses studied both in preclinical settings and in clinical trials. Lastly, the potential limitations and safety issues will be discussed that will, for a large part, determine whether oncolytic viral therapy can become a standard cancer therapy in the future.

Mechanisms of tumor-selective replication

Naturally occurring oncotropic viruses

Since the cellular changes induced by viral infection are often strikingly similar to cellular changes acquired during carcinogenesis (for example, inactivation of p53, inhibition of apoptosis, and induction of mitosis), it is not surprising that given this genetic convergence many viruses preferentially grow in tumor cells. Several of these naturally occurring oncotropic viruses are used for oncolytic virotherapy (Table 1).

Reovirus. This virus is a nonenveloped double-stranded RNA virus with minimal pathogenicity in humans.² Although its preferential tropism and cytotoxicity for neoplastic cell lines has been acknowledged for a long time,¹³ the underlying mechanism had not been elucidated until recently. Mouse fibroblasts, which are normally resistant to reovirus infection, become susceptible to infection following transformation by activation of the ras-pathway, which is often constitutively activated in tumors.^{14,15} In addition, Strong et al¹⁶ demonstrated that deletion of the gene coding for double-stranded RNA-

activated protein kinase (PKR), which is a key component of the intracellular host defense system against pathogens like viruses, allows reovirus infection and replication. These findings implicate that reovirus, as it is not able to directly block the antiviral effects of PKR, as some other viruses do (see below), depends on activated ras-pathways in order to circumvent the effect of PKR (see Figure 1).

Given the fact that approximately half of the tumors bear an activating mutation in the ras-pathway,¹⁷ reovirus might be an ideal oncolytic agent. Reovirus has been shown to destroy selectively many different types of neoplastic cells, including those derived from breast, colon, ovaries, and lymphoid malignancies both *in vitro* and in xenograft mice models both intratumoral (i.t.)^{14,18–21} and intravenously (i.v.).¹⁸ However, in these preclinical studies, immunodeficient mice were used, in which the oncolytic effect of the virus is not affected by possible antiviral immune responses. Reovirus treatment combined with immune suppression (cyclosporine A or anti-CD4/anti-CD8 antibodies) further enhanced the oncolytic effects.²² Altogether, these results will likely inspire further investigation into the potential use of reovirus as an anticancer agent.

Newcastle disease virus (NDV). This virus is an enveloped chicken paramyxovirus that carries its genetic information on a negative single-stranded RNA molecule. The virus is not pathogenic to humans and it was first reported to have oncolytic activity in the mid-1950s.²³ There are indications that NDV similar to reovirus is dependent on an activated ras-pathway to be able to replicate efficiently.²⁴ Defects in the IFN-pathway, which are often found in tumor cells, might also render these cells NDV sensitive.

A strain of NDV, 73-T, so named because it was passaged 73 times through mouse ascites cells *in vitro* has

Table 1 Natural tumor selective viruses

Virus	Class, Subclass	Genetic/phenotypic target within tumors	Advantage	Disadvantage
NDV	ssRNA ⁻ , rhabdovirus	Activated ras-pathway; defective IFN-pathway	Infects primarily tumor cells Not pathogenic in humans Antigenicity generates immune response Does not establish permanent infection in host	Transgene insertion reduces viral replication
Reovirus	dsRNA, reovirus	Activated ras-pathway	Mild pathogenicity Unable to infect normal cells Antigenicity generates immune response	Infects only cells with activated ras-pathway
Autonomous parvovirus	ssDNA, parvovirus	IFN-resistance of tumors	Unable to infect nondividing cells No pathogenicity	Not very effective in inducing cell lysis
VSV	ssRNA ⁻ , rhabdovirus	Loss of cell cycle control?	Unable to infect normal cells Very high replication rates	Infects only cells with defective IFN-pathway Very high replication rates

NDV, Newcastle disease virus; VSV, vesicular stomatitis virus; IFN, interferon; ds, double stranded; ss, single stranded; ssRNA⁻, RNA as a template for mRNA.

Figure 1 Role of PKR in viral infection. PKR is an important downstream effector molecule of the interferon (IFN) signalling pathway. The IFN signalling system is part of the host defense system against pathogens like viruses. When a cell is infected by a virus, it is stimulated to produce IFNs that will activate an antiviral defense response of nearby cells. Upon binding of IFNs to cell surface receptors (IFN-Rs) on neighboring cells, an intercellular signal transduction cascade is engaged, that induces PKR expression. When PKR, subsequently, encounters viral double-stranded (ds) RNA, PKR will bind to the RNA. This event leads to autophosphorylation of the PKR homodimer and activation of its kinase activity. Activated PKR phosphorylates eIF-2 α , which in turn inhibits protein synthesis and thereby viral replication. However, in tumor cells this pathway is often deregulated. For example, in many tumor cells inactivating mutations of the IFN signalling pathway can be found (VSV is dependent on this characteristic for its natural oncotropism). In addition, a constitutively activated ras signal transduction pathway, which is a commonly found feature in tumor cells, leads to inhibition of PKR autophosphorylation (for example reovirus is dependent on this characteristic for its natural oncotropism). On the other hand, viruses have also developed mechanisms themselves to inhibit this antiviral response. Adenovirus can produce so-called virus-associated (VA) RNAs that bind to PKR leading to inactivation of it. Furthermore, herpesvirus is able to express the protein γ 34.5. By interacting with cellular phosphatase 1 α (PP1 α), γ 34.5 dephosphorylates eIF-2 α and induces host protein synthesis needed for viral replication (GF = growth factor, EGFR = epidermal growth factor receptor).

been shown to induce significant tumor regression in several different tumor lines and mouse models bearing fibrosarcoma, neuroblastoma, colon, prostate, large cell lung or breast carcinoma xenografts.^{24,25} Apart from its use as a direct oncolytic virus, it has also been studied as part of a viral oncolysate, which is a suspension of virus and tumor cells, as a mode for cancer therapy.²⁶

Another naturally attenuated strain of NDV, designated PV701, also exhibits a high potency and tumor selectivity against a broad range of human tumors as 80% of human cancer cell lines are two to four log orders more sensitive than normal cells to PV701-mediated killing. Furthermore i.t. treatment of several human tumor xenografts caused high rates of tumor regression in a mouse model (reviewed by Mullen and Tanabe²⁷). The encouraging results have led to the initiation of a phase I trial of i.v. administration of PV701 in patients with advanced solid cancers. Little toxicity was reported even at high doses, and two partial responses occurred at high dose levels.²⁸ Additional clinical studies are now underway.

Vesicular stomatitis virus (VSV). This virus is an enveloped negative single-stranded RNA rhabdovirus

and is primarily of veterinary importance. However, the virus has been shown to replicate more efficiently in transformed cell lines relative to nontransformed cell lines.²⁹

VSV is known to be exquisitely sensitive to IFN-mediated antiviral effects and is dependent on cells that have a malfunctioning IFN-pathway to make replication possible.²⁹ Mutations causing defects in the IFN-pathway are often found in tumor cells, because apart from its antiviral effects (Fig 1), the IFN-mediated signal cascade can also lead to growth inhibitory and apoptotic signals. As a result, these tumor cells with mutated IFN-pathways may have a growth and/or survival advantage, but are at the same time more susceptible to viral infections like VSV.

In vivo work showed that in athymic mouse models i.t. injection of VSV was effective in reducing the tumor size of subcutaneous human melanoma xenografts.²⁹ Treatment of VSV in an immune-competent mouse model of metastatic colon cancer, resulted in complete and durable cures in the majority of treated animals.³⁰ Furthermore, the survival of multifocal hepatocellular carcinoma was significantly prolonged after administration into the hepatic artery.³¹ Although these results are encouraging, further research is needed to find out whether VSV can be

used as an oncolytic tool for the treatment of interferon nonresponsive tumors.

Autonomous parvovirus. This virus is a small nonenveloped single-stranded DNA virus. Unlike other DNA viruses, it is unable to force resting cells into S phase and is therefore incapable of undergoing genome replication in quiescent cells. Therefore, the efficiency with which parvoviruses replicate in transformed cell lines relative to nontransformed cell lines is probably due to the aberrant cell cycle control exhibited by transformed cells (reviewed by Rommelaere and Carnelis³²). Although autonomous parvovirus has proven to have antineoplastic activity *in vitro*,³³ it is often not potent enough to kill all tumor cells equally well.^{32,34,35} Delivery of cytotoxic compounds to tumor cells by parvoviral vectors, was demonstrated to enhance the intrinsic antitumor effect of autonomous parvovirus in human tumor cell cultures.^{36,37} Results from *in vivo* studies will determine whether there is a possible application of recombinant autonomous parvoviruses as a therapy for some human cancers.

Engineered tumor-selective viruses

Viruses are also being evaluated that are genetically engineered for tumor selectivity. The main approach by which viruses can be made tumor selective is by modification of the cellular tropism at the level of viral

replication in a way that it becomes dependent on specific characteristics of tumor cells for viral replication. This can either be achieved by deleting viral genes that are critical for viral replication in healthy cells but are dispensable upon infection of neoplastic cells (Table 2), or, on the other hand, by placement of tumor-specific promoters upstream of viral genes that are critical for efficient viral replication (Table 3). Modification of the cellular tropism at the level of cell recognition and binding has also been performed by altering the viral coat for tumor-selective binding and uptake. However, this strategy has mainly been applied to replication deficient viruses and for that it falls beyond the scope of this review and we would like to refer to other papers.^{38–40}

Viral gene deletion mutants

Adenovirus gene deletion mutants. One of the most extensively studied viruses that has been genetically engineered for oncolytic purposes is adenovirus, in particular serotypes 2 and 5. It is a nonenveloped, linear, double-stranded DNA virus with a genome size of approximately 38 kb. It is endemic in the human population and its natural pathogenicity is associated only with mild respiratory infections. In order to replicate, adenoviruses promote entry into the cell cycle of the host cell through expression of the E1A and E1B. E1A competes with E2F for binding to pRb, resulting in release of transcription factor E2F and subsequent transition from G1 into S phase. In addition,

Table 2 Examples of viral gene deletions to achieve tumor-selective replication

Parental strain	Agent	Genetic alteration	Genetic/phenotypic target within tumours	In clinical trials	References
Adenovirus	ONYX-015	E1B-55kd deletion	p53 null/mutant or/and inactivated p53-pathway	I–III	41
	Ad-Δ24	E1A CR2 deletion	pRb null/mutant or/and loss of cell cycle control	No	60
	CB-1	E1A CR2 deletion; E1B-55kd deletion	pRb and p53 defective pathways or/and loss of cell cycle control	No	61
	d1922-947	E1A CR2 deletion	pRb null/mutant or/and loss of cell cycle control	No	59
	d1331	VA-1 gene deletion	Ras-activated pathway	No	62
	d1sptk	Thymidine kinase gene deletion	Replication	No	12
Herpes simplex virus-1	R3616	Deletion of both γ 34.5 genes	Loss of neurovirulence	No	65
	HrR3	ICP6 gene deletion	Replication	No	67
	G207	Deletion of both γ 34.5 genes; ribonucleotide reductase disruption	Replication; loss of neurovirulence	I–II	69
	NV1020	Deletion of one γ 34.5 gene; deletion in tk gene; insertion of exogenous copy of tk gene	Replication, loss of neurovirulence	I	72
Vaccinia virus	VV-TK ⁻	Thymidine kinase gene deletion	Replication	No	75
	VV-SPI-1/2 ⁻	Deletion of SPI-1 and SPI-2 genes	Replication	No	27
	WDD	Thymidine kinase gene deletion; deletion of VGF gene	Replication	No	76
Polio virus	PV1(RIPO)	IRES element replaced by IRES from HRV2	Loss of neurovirulence; replication	No	77
Influenza virus	IVA-NS1 ⁻	NS1 gene deletion	IFN-pathway deficiency	No	78

tk, thymidine kinase; IRES, internal ribosomal entry site; HRV2, human rhinovirus type 2; IFN, interferon.

Table 3 Examples of tissue- and tumor-specific promoters used for tumor-selective replication of oncolytic viruses

Promoter	Tissue/tumour type	Applied virus	References
Osteocalcin promoter	Osteosarcoma	Adenovirus	82
PSA promoter	Prostate	Adenovirus	79,81
AFP promoter	Hepatocellular carcinoma	Adenovirus	84,85
Tyrosinase promoter	Melanocytes	Adenovirus	87
MUC-1 promoter	Breast carcinomas	Adenovirus	86
		HSV-1	102
Midkine differentiation factor promoter	Neuroblastoma	Adenovirus	90
Rat probasin promoter	Prostate	Adenovirus	81
Leukoprotease inhibitor promoter	Ovarian carcinoma	Adenovirus	89
Tcf responsive promoter	Colon cancer	Adenovirus	91
		Autonomous parvovirus	103
Calponin promoter	Smooth muscle cells	HSV-1	101
Albumin enhancer/promoter	Liver	HSV-1	99
Flt-1 promoter	Teratocarcinoma	Adenovirus	96
E2F-1 promoter	Cancer cells in general	Adenovirus	93
Telomerase reverse transcriptase promoter	Cancer cells in general	Adenovirus	94,95
Hypoxia responsive elements	Cancer cells in general	Adenovirus	97

PSA, prostate-specific antigen; AFP, α -fetoprotein; Flt-1, vascular endothelial growth factor receptor type 1; HSV-1, herpes simplex virus-1.

Figure 2 Cell cycle control mechanisms. The retinoblastoma protein family (pRb) regulate the G1 to S-phase cell cycle checkpoint. When pRb is hypophosphorylated it prevents progression of the cell cycle from G1 to S phase. pRb exerts this effect by binding to and repressing transcription factor E2F, which normally induces expression of genes needed for DNA synthesis. When a cell receives a mitogenic signal, one of the upregulated proteins is cyclin D, which subsequently enables cyclin-dependent kinase 4 (CDK4) to phosphorylate pRb. As a result of this phosphorylation, pRb is not able to bind to and repress E2F anymore, leading to progression into the S phase and subsequent cell cycle phases. Another protein that is of great importance with regard to cell cycle control is tumor suppressor protein p53. p53 is a transcription factor that is upregulated and activated upon stress signals like DNA damage or viral infection. p53 can activate transcription of genes coding for proteins that either induce apoptosis (for example Bax) or cell cycle arrest (for example p21). p21 is able to inactivate CDKs, thereby inhibiting progression through the cell cycle. The activity of p53 is counteracted by Mdm2, that inactivates p53 through binding and subsequent ubiquitin mediated degradation. In its turn, the effect of Mdm2 can be inhibited by p14^{ARF}, a tumor suppressor protein that is upregulated by stress signals and mitogenic signals, like E2F. As E2F activity is blocked by pRb, inactivation of pRb leads to activation of p53 and functions as a safety mechanism during progression through the cell cycle. Altogether, it is not surprising that inactivation of the critical pRb or p53 pathways by either mutations (as in tumors) or intervention by viral proteins, like adenoviral E1A and E1B-55kd, will lead to loss of cell cycle control (reviewed by Sherr¹⁶²).

E1B-55kd is able to bind and inactivate p53. If it were not for E1B-55kd expression, cellular levels of p53 would rise in response to adenoviral infection with resulting apoptosis or cell cycle arrest attenuating viral replication (Fig 2). Nearly all tumors possess defects in their p53 and/or pRb pathways. Thus, E1A and E1B gene products become dispensable in these

tumor cells, as E1A- or E1B-defective adenoviruses can still replicate in pRb- or p53-defective tumor cells, respectively. In contrast, replication of these mutated viruses is attenuated in normal cells with intact p53 and pRb pathways.

The first conditionally replicative adenovirus (also termed CRAd), ONYX-015 (also called *d11520* or CI-1042)

was reported in 1996.⁴¹ ONYX-015 is a type 2, type 5 chimeric adenovirus that contains alterations in the E1B gene, resulting in the complete abrogation of E1B-55kd expression. It was demonstrated that ONYX-015 efficiently replicated only in cells lacking functional p53 and it caused significant tumor regression in mice bearing human tumor xenografts, due to tumor-specific viral replication.^{41,42} In spite of this enthusiasm, there have been reports contradicting the mechanism of ONYX-015 selectivity, based on findings that cells with functional p53 also support its viral replication.^{43–45} Several hypotheses have been put forward to explain this apparent contradiction. First, p53 function can be abrogated in tumors by mechanisms other than p53 gene mutations. Loss of function of p14^{ARF}, which normally indirectly stabilizes p53, can result in inactivation of p53 (Fig 2). Ries et al⁴⁶ showed that loss of p14^{ARF} in tumor cells expressing functional p53 facilitates replication of ONYX-015. Second, adenoviral protein E4orf6, which is still present in ONYX-015, is known to bind and inactivate p53 as well and may in part compensate for E1B-55kd loss.⁴⁷ The exact role of p53 status in ONYX-015 infection remains to be elucidated.

The safety and antitumor efficacy of ONYX-015 have been tested in numerous phase I and II clinical trials with multiple tumor types and several routes of administration (for example, intratumoral, intravenous, intra-arterial, and intraperitoneal). In all cases, safety was demonstrated even at doses up to 2×10^{12} particles, with most common associated toxicity being flu-like symptoms. Viral replication was tumor selective after administration by all routes, but the local tumor regression was modest, 0–14% (reviewed by Kirn⁴⁸). The most encouraging results were gained from phase I and II studies using i.t. administration of ONYX-015 in patients with advanced head and neck squamous cell carcinoma.^{49–51} Treatment was well tolerated at the highest dose level (10^{11} PFU) and the virus was shown to replicate in tumor cells but not in peritumoral tissue.⁵¹ Hyperfractionated treatment resulted in 10% complete response, 62% stable disease, and 29% progressive disease rates. Importantly, the presence of high neutralizing antibody titers, which is normal during adenoviral infection, did not prevent infection and/or replication within tumors.^{49,50} However, other clinical trials were less promising. With i.t. treatment of pancreatic tumors or ovarian carcinoma no objective tumor responses were observed.^{52,53} Phase I/II studies in patients with unresectable primary and secondary liver tumors⁵⁴ and colorectal carcinoma liver metastases,^{55,56} showed that intra-arterial administration of ONYX-015 was well tolerated, but did not result in significant tumor regression. On the other hand, intravenous treatment with ONYX-015 did appear least promising, with no evidence of antitumor activity in patients with advanced carcinoma metastatic to the lung⁵⁷ and stable disease in some patients with metastatic colorectal cancer but only for a few months.⁵⁸ Although ONYX-015 seems not to be very successful in most cancers as monotherapy, it does hold promise in combination with other therapies like chemotherapy, radiation therapy and

gene therapy (see ‘Oncolytic Virotherapy in a multimodal antitumor approach’ section).

Another strategy that has been used to make adenoviruses tumor selective is by mutation of the E1A gene. E1A mutant adenovirus, *d1922-947*, with a small deletion in conserved region 2 (CR-2) of E1A was shown to replicate specifically in cancer cells with abnormalities in the pRb-pathway and to reduce the incidence of metastases in a breast tumor xenograft model following intravenous injection.⁵⁹ Another E1A mutant, termed $\Delta 24$, carrying a 24-bp deletion in CR-2 responsible for pRb binding, displayed efficient replication in pRb-lacking cells, while infection of cells with functional pRb was strongly attenuated.⁶⁰ *In vivo*, multiple injections induced 83.8% inhibition of tumor growth in nude mice.

These E1A virus mutants have not yet been tested in clinical trials, but are more promising as antitumor agent than ONYX-015, since *d1922-947* demonstrated significantly greater potency compared to ONYX-015 both *in vitro* and *in vivo*.⁵⁹ The reduced potency of ONYX-015 might be due to the loss of p53-independent E1B-55kd functions. E1B-55kd also plays a role in viral mRNA transport.⁴⁵ Abolishment of this function as well, is likely to reduce viral replication efficacy. In contrast, the E1A deletions of *d1922-947* and $\Delta 24$ only abolish its binding capacity to bind pRb, leaving other functions of the E1A gene in tact. In a recent study a novel adenovirus mutant (CB1) harboring targeted deletions in both E1 genes was successfully tested for its oncolytic properties as it induced a potent antiglioma effect both *in vitro* and *in vivo*.⁶¹

Yet, a recent study used another mechanism to make adenovirus tumor selective. Adenovirus produces so-called virus-associated (VA) RNAs that bind to PKR and inactivate it. Viruses (for example reovirus) that are unable to counteract the effect of PKR, replicate to higher levels when PKR is inhibited by activated ras-pathways. Since activated ras-pathways are often found in tumors, adenovirus mutants that are unable to produce VA RNAs can be rendered tumor selective (Fig 1). Cascallo et al⁶² demonstrated ras-dependency of a VA RNA mutant for replication and might be of use in therapy of tumors with constitutively activated ras-pathways, like pancreatic tumors.

HSV-1 gene deletion mutants. Besides adenovirus, herpes simplex virus 1 is one of the most extensively studied viruses for oncolytic virotherapy. HSV-1 is an enveloped, double-stranded DNA virus with a genome size of approximately 152 kb and is generally neurotropic. It rarely produces severe medical illness in immunocompetent adults. It is an attractive virus for gene therapy, since as much as 30 kb of its genome may be replaced for delivery of transgenes.

The utility of gene deletion mutants was first demonstrated with HSV in 1991.¹² In this mutant, termed *Δlspk*, the thymidine kinase gene (HSV-tk) was deleted, which is normally needed for nucleic acid metabolism. For its replication, this mutant is dependent on endogenous levels of this enzyme, which is only found in high concentrations in replicating cells. Accordingly, *Δlspk*

replicated selectively in glioma cell lines and i.t. administration caused growth inhibition of U87 human glioma in both nude¹² and immunocompetent mice.⁶³ However, apart from infection of tumor cells, healthy neurons were also infected and neurotoxicity was found.⁶⁴ In addition, these mutants are resistant to antiviral agents acyclovir and ganciclovir by virtue of their disrupted HSV-tk gene. For safety reasons, sensitivity to agents that can block unwanted replication is desirable. Therefore, investigators have come up with other HSV-1 mutants that are still sensitive to antiherpetics and show less neurovirulence.

One such mutated virus harbors mutations in both γ 34.5 loci⁶⁵ (Fig 3). The protein product of γ 34.5 blocks the shut-off of host protein synthesis in infected cells by interacting with cellular phosphatase 1 α (PP1 α) to dephosphorylate eIF-2 α . This leads to production of more progeny viruses from each infected cell (Fig 1). Due to specific deletions in this gene, these HSV mutants are strongly attenuated for their replication in normal cells, like neurons, leading to loss of their neurovirulence.^{65,66} However, γ 34.5 mutated HSV-1 replicated in actively dividing cells, like tumor cells. Moreover, these mutants were still sensitive to antiherpetics, as the HSV-tk locus is not disrupted. Yet, another genetically engineered virus has been produced, that contains mutations in the ICP6 gene, normally encoding the large subunit of ribonucleotide reductase, needed for generation of deoxyribonucleotides for DNA synthesis⁶⁷ (Fig 3). ICP6-mutated viruses replicate only in actively dividing cells that provide ribonucleotide reductase in complementation, rendering them tumor selective. Drug sensitivity assays showed that this ICP6 mutant virus was hypersensitive to ganciclovir and showed significant inhibition of tumor growth after

i.t. administration in mice bearing human glioblastoma tumors⁶⁷ or colon carcinoma.⁶⁸

More recently, herpes viruses have been engineered that contain mutations in more than one gene. The major advantage of this multiple mutation approach relative to single gene mutations is the reduction of chance of reversion to wild-type strains, so providing an extra level of safety. A promising HSV-1 mutant is G207,⁶⁹ which has deletions in both γ 34.5 loci and an inactivating insertion in the ICP6 gene (Fig 3). G207 is not neurovirulent and is sensitive to antiherpetics. Furthermore, G207 exhibits tumor cell-specific replication and antitumor efficacy in both *in vitro* and *in vivo* models of malignant gliomas,⁶⁹ colorectal cancers, ovarian cancer, breast cancer and prostate cancer (reviewed by Varghese and Rabkin⁷⁰). A phase I clinical dose escalation study of G207 in the treatment of recurrent malignant glioma was initiated and no toxicity was found, even at the highest doses (3×10^9 PFU).⁷¹ Unfortunately, no viral replication data are available, but preparations for phase II trials are underway. NV1020 is another genetically engineered oncolytic herpes virus that contains a deletion in the endogenous HSV-tk gene as well a deletion in one of the two γ 34.5 genes (Fig 3). The NV1020 genome, however, does harbor an exogenous copy of the HSV-tk gene derived from HSV-2. Because of this insertion, NV1020 maintains its sensitivity to acyclovir and ganciclovir. NV1020 was first designed as a candidate for human immunisation against infections with HSV-1 and HSV-2.⁷² After NV1020 was demonstrated to be a safe virus in extensive rodents and primate studies, it was tested for its oncolytic potency in preclinical models of pancreatic carcinoma, transitional cell carcinoma, head and neck squamous cell carcinoma, hormone resistant prostate

Figure 3 Map of the genome of herpes simplex virus. The HSV-1 genome is a single, linear molecule of double-stranded DNA approximately 152,000bp in length. It is divided into two unique segments called long (U_L) and short (U_S). Short regions of repeated sequence (a/b/c and a'/b'/c') occur at the genome ends and between the L and S segments. As DNA is replicated, the L and S segments invert at a high rate creating a total of four genome isomers. The four occur at equal frequencies in most wild-type HSV-1 populations. The genome encodes 74 proteins. Most genes encoding proteins are located in the L or S regions, and they are named according to their location within L or S. For example, the *US6* gene encodes glycoprotein D, a membrane glycoprotein involved in virus entry, and *UL30* encodes the virus DNA-dependent DNA polymerase. HSV-1-encoded proteins can be divided into four main categories based on their function: proteins for (1) virus DNA replication and recombination; (2) transcriptional regulation of HSV-1 gene expression and modulation of the host cell; (3) capsid formation and assembly; and (4) proteins that are embedded in the envelope. A schematic representation of the positions of the genes encoding these proteins in the HSV-1 genome is depicted above. Genes that are modified or deleted to achieve tumor-specific targeting and replication are indicated as well.

carcinoma, and epidermoid carcinoma (reviewed by Varghese and Rabkin⁷⁰). In these studies NV1020 replicated selectively in tumor cells in high titers and caused rapid tumor regression. Comparison of NV1020 with G207 revealed that NV1020 is capable of more efficient viral replication than G207 *in vitro*⁷³ and that the oncolytic efficacy of NV1020 was superior to G207 at lower concentrations of the virus both *in vitro* and *in vivo*.⁷⁴ Given these quite promising results, a phase I trial of intrahepatic arterial injection of NV1020 in patients with colorectal carcinoma liver metastases was recently initiated. Despite the promising results obtained with engineered HSV-1 vectors described thus far, it is likely that multimodal approach to eradication of cancer will be more effective (see the later section).

Vaccinia virus gene deletion mutants. A virus that has just recently come into the picture as an agent for oncolytic virotherapy is vaccinia virus. Vaccinia virus is an enveloped, double-stranded virus with a genome of approximately 200 kb. This large genome size makes it a promising vector as it allows insertion of large genes for virotherapeutic approaches. It is best known as the first widely used vaccine, which resulted in the eradication of smallpox. As a result, its toxicity is well characterized. Until recently, the use of vaccinia virus as an anticancer agent has primarily focused on the induction of an antitumoral immune response, since it is highly immunogenic (see 'Eliciting an antitumor immune response' section). However, recently, investigators have generated vaccinia virus mutants that are replication-conditional such that they destroy cancer cells as a by-product of viral replication in a way that they become oncolytic. The most commonly employed strategy is analogous to the one used in HSV-1 mutants: inactivation of the thymidine kinase (tk) gene. Puhlmann et al⁷⁵ showed that tk-deficient, replication competent vaccinia virus mutants are selectively oncotropic with several logs higher replication kinetics in tumor cells relative to normal cells. To enhance the tumor specificity of this vector, a combined tk- and vaccinia growth factor-deleted vaccinia virus was developed.⁷⁶ This mutant vaccinia virus was significantly attenuated in resting cells *in vitro* and demonstrated significant tumor regression after systemic administration with 1×10^9 PFU in nude mice bearing murine colon adenocarcinoma. Furthermore, a mutant vaccinia virus with improved tumor selectivity has been generated by the deletion of SPI-1 and SPI-2 genes. These viral genes encode serine protease inhibitors that are homologous to proteins known to be upregulated in cancer and so are dispensable upon infection of tumor cells but are required for successful viral replication in normal cells (reviewed by Mullen and Tanabe²⁷). Most of these mutant vaccinia viruses have been developed for enhanced targeting of therapeutic genes to tumors (see Table 4). Apart from its role in anticancer virotherapy as an efficient gene delivery vector, these studies also raise the possibility of the use of vaccinia virus as direct oncolytic agent in the fight against cancer.

Poliovirus gene deletion mutants. A recent study reported an attenuated poliovirus as a potential antitumoral agent.⁷⁷ Poliovirus is a nonenveloped positive-single-stranded RNA virus, causing the neurodegenerative disease polio, which is now due to the development of an effective vaccine almost completely under control. Translation of the viral noncapped RNA is dependent on a cell type-specific internal ribosomal entry site (IRES) element. Exchange of poliovirus IRES with its counterpart from human rhinovirus type 2 resulted in attenuation of neurovirulence in primates. Despite deficient virus propagation in cells of neuronal origin, nonpathogenic polio recombinants retained excellent growth characteristics in cell lines derived from glial neoplasms and demonstrated strong oncolytic potential against experimentally induced gliomas in athymic mice. These observations suggest that highly attenuated poliovirus recombinants may have antineoplastic effects. Unlike the other viruses described above, this virus contains genetic material derived from two different virus types. The *in vivo* characteristics, like host cell range and pathogenicity, might therefore have been significantly altered. Thus, it will be of great importance to carefully address these safety issues, before any other studies concerning its clinical relevance can be undertaken.

Influenza virus gene deletion mutants. Recently, a recombinant influenza A virus, normally causing common cold, has been engineered, that lacks the NS1 protein. Since the NS1 protein of influenza virus is a virulence factor that counteracts IFN-mediated antiviral response by the host, this modified virus is dependent on cells with defective IFN pathways, which is often the case in tumors, for efficient replication. The oncolytic potency of NS1-modified influenza virus was demonstrated both in IFN-resistant tumor cells and in a SCID mouse model of a subcutaneously implanted human IFN-resistant melanoma.⁷⁸

Viral dependency on tissue/tumor-specific promoters

Tissue/tumor-specific promoters constructed into adenovirus. A number of oncolytic adenoviruses have been generated in which expression the early genes E1A, E1B, E3 or/and E4 are under control of exogenous promoters that are preferentially active in tumor cells (see Table 3).

Most research concerning the application of tissue-specific promoters for tumor-specific adenoviral replication has been carried out on prostate cancer. One virus that has been yielded from this line of investigation is CV706. In this CRAd the prostate-specific antigen (PSA) gene promoter-enhancer element is inserted upstream of the E1A gene, which is needed to induce the host cell to enter the cell cycle, thereby allowing viral replication to ensue. PSA is a widely used marker for the diagnosis and management of prostate cancer. Replication kinetics of CV706 were highest in PSA-producing tissues and CV706 was able to destroy large PSA-expressing tumors in an i.t. xenograft mouse model.⁷⁹ In addition, Deweese et al⁸⁰ demonstrated in a phase I trial that intraprostatic delivery

of CV706 was safe at high doses and treatment resulted in strong reduction of serum PSA levels. Another prostate-specific oncolytic adenovirus, termed CV787, contains the prostate-specific rat probasin promoter driving E1A expression and the human PSA gene promoter–enhancer element driving the E1B gene. This virus, unlike CV706, contains a wild-type E3 region, which encodes proteins that play a role in viral progeny release and cell lysis (see also Table 4). The presence of a functional E3 region significantly improved cell killing and oncolytic efficacy.⁸¹ CV787 was shown to destroy PSA⁺ cancer cells 10⁴ times more efficiently than PSA[−] cells. In SCID mice carrying PSA⁺ tumor xenografts, a single i.v. injection of CV787 eliminated tumors within 4 weeks.⁸¹ CV787 is currently being studied in clinical trials and results concerning viral safety are underway. Prostate cancer has a high propensity to metastasize to bone, and often resists hormone, radiation, and chemotherapies. Therefore, Matsubara et al⁸² used the osteocalcin promoter to target adenovirus to metastasized prostate cancer cells. In SCID mice, intraosseous growth of PSA-producing xenografts responded markedly to i.v. administrations of a single dose of the virus. In all, 100% of the treated mice responded to this systemic therapy with a decline of serum PSA to an undetectable level. These promising results have led to the initiation of clinical trials and are now underway.⁸³

In addition to prostate cancer-specific adenoviruses, a range of oncolytic adenoviruses have been engineered that

target other tumors based on construction of tissue-specific promoters upstream of E1A. Several studies have reported construction of an adenovirus whose E1A is expressed under control of the tumor-specific α -fetoprotein (AFP) promoter. AFP is highly expressed in up to 80% of patients with hepatocellular carcinoma but not in normal adults. Specific replication and lysis was observed in AFP-expressing cell lines, while replication in AFP[−] cells was strongly attenuated.^{84,85}

Similar strategies were used to target adenoviruses to breast carcinomas, using the DF3/MUC1 gene promoter;⁸⁶ to melanoma cells, using a human tyrosinase enhancer/promoter;^{87,88} ovarian carcinoma, using a secretory leukoprotease inhibitor promoter;⁸⁹ neuroblastoma, using the midkine differentiation factor promoter;⁹⁰ and colon cancer, using a Tcf responsive promoter.⁹¹ In colon cancer cell lines, it was demonstrated that tumor-selective viral replication was further enhanced when multiple early genes (E1A E1B, E2 and/or E4) were under regulation of Tcf-responsive elements.^{91,92}

Although viruses harboring tissue-specific promoters have been shown to preferentially replicate in tumor cells, they are limited in that they are only targeted to specific subsets of tumor types. In addition, as these promoters are tissue specific and therefore not necessarily only active in tumors, this approach might lead to some degree of viral replication in tissues other than tumors alone. To overcome this limitation, the use of promoters that are

Table 4 Strategies to improve the intrinsic anti-tumor potency of oncolytic viruses

Strategy	Example	Way of action	Applied virus (inserted gene)	References
1. Insertion of genes encoding cytotoxic proteins	Expression of adenoviral E3 gene coding for adenoviral death protein	Increase of viral-induced cell lysis	Adenovirus (E3)	81,105
	Expression of syncytium-inducing genes	Increase of cell killing of both infected and uninfected cells through syncytium induction	Adenovirus (FMG)	109
	Deletion of apoptosis inhibitor E1B-19kd protein	More efficient cell killing by viral-induced apoptosis	HSV-1 (FMG) Adenovirus (—)	110 106,108
2. Elicitation of an antitumor immune response	Expression of cytokine genes	Activation and stimulation of tumor-specific cytotoxic T cells	HSV-1 (IL-4)	120
			HSV-1 (IL-12)	119,121,163
			Vaccinia virus (GM-CSF)	122
			Vaccinia virus (IL-2)	123
			Parvovirus (MCP-1)	37
			Adenovirus (IFN)	118
			Vaccinia virus (—)	113
	Use of oncolysates: treatment with virus-augmented tumor cells	Elicitation of an immune response directed against tumor-specific antigens	VSV (—) NDV (—)	115 114
	Expression of tumor-specific antigens	Elicitation of an immune response directed against tumor-specific antigens	Vaccinia virus (CEA)	117
			Vaccinia virus (PSA)	116

HSV-1, herpes simplex virus-1; NDV, newcastle disease virus; VSV, vesicular stomatitis virus; GM-CSF, granulocyte–macrophage colony-stimulating factor; MCP-1, monocyte chemotactic protein 1; FMG, fusogenic membrane glycoprotein; IL, interleukin; IFN, interferon; CEA, carcinoembryonic antigen; PSA, prostate-specific antigen.

specifically active in tumors has been explored. All tumor cells exhibit uncontrolled cell growth due to a deregulated cell cycle generally characterized constitutively active E2F (Fig 2). Based on this observation, an adenovirus has been engineered that contains a human E2F-1 promoter element driving E1A expression. It selectively induced cell lysis of tumor cells while normal noncycling cells were unharmed and showed potent antitumor effects in an ovarian cancer xenograft mouse model.⁹³

The telomerase promoter has also been explored as means to direct viral replication to tumor cells. Telomeres become shorter during each cell cycle, limiting the number of cell divisions. The enzyme telomerase is able to lengthen these telomeres, and its expression is a common characteristic of most solid tumors. A CRAd has been developed, which contains a promoter element of human telomerase reverse transcriptase driving E1A expression. Selective cytolysis of telomerase-positive cells was observed and administration of the viral mutant to tumor xenografts resulted in significant inhibition of tumor growth.^{94,95}

Growth factor receptors are also often upregulated in tumor cells. One study showed that an adenovirus mutant, containing the E1 gene under control of a vascular endothelial growth factor receptor (flt-1) promoter, was specifically targeted to teratocarcinoma cell lines.⁹⁶ Another option currently being evaluated is the use of hypoxia responsive elements (HREs), as hypoxia is a common hallmark of solid tumors. Hypoxia-inducible factors (HIFs) mediate transcriptional responses to hypoxia by binding to these HREs in target genes. HYPR-Ad#1, containing an HIF-responsive HRE in the promoter of E1A, caused conditional cytolysis of hypoxic/HIF activated but not normoxic cells.⁹⁷

Several second-generation CRAds have been developed that combine transcriptional targeting with viral gene mutations in order to further restrict viral replication to tumor cells. For example, Nettelbeck et al⁸⁷ have engineered a melanoma targeted CRAd, AdTyrΔ24, in which the human tyrosinase enhancer/promoter is used to drive expression of E1A genes containing mutations that abrogate binding to proteins of the pRb family. Likewise, a viral mutant harboring the promoter of surfactant protein B upstream of E4 coupled with small attenuating deletions in the E1A gene has been demonstrated to specifically replicate in alveolar and bronchial tumor cells.⁹⁸

Tissue/tumor-specific promoters constructed into HSV-1. This strategy of using tissue or tumor-specific promoters to target oncolytic viruses to tumor cells has also been applied on HSV-1 vectors. G92A is one such vector, in which an albumin enhancer/promoter - ICP4 transgene is inserted into the thymidine kinase gene of HSV-1 mutant *d120*, deleted for both copies of the ICP4 gene⁹⁹ (Fig 3). In adults, albumin is exclusively expressed in the liver, and it was shown that G92A replicated 1000 times more efficiently in hepatoma cells than in other nonalbumin-expressing cells. *In vivo*, G92A replicated well in subcutaneous xenografts of human hepatoma cells

in athymic mice, but not in nonhepatoma subcutaneous tumors. I.t. inoculation of G92A inhibited the growth of established subcutaneous hepatoma tumors in nude mice, but not prostate tumors.¹⁰⁰ Another HSV-1 mutant has been engineered, using the calponin promoter to drive expression of ICP4 to target viral replication.¹⁰¹ Normally, calponin is expressed in matured smooth muscle cells, but is aberrantly expressed in a variety of malignant soft tissue and bone tumors. In cell culture, the virus selectively killed calponin-positive human synovial sarcoma, leiomyosarcoma, and osteosarcoma cells. Furthermore, in a leiomyosarcoma xenograft nude mouse model i.t. viral treatment resulted in total tumor regression in four out of five mice. More recently, it was demonstrated that insertion of the DF3/MUC1 promoter/enhancer sequence in front of the γ 34.5 locus resulted in effective eradication of DF3/MUC1⁺ carcinoma xenografts in nude mice.¹⁰²

Tissue/tumor-specific promoters constructed into autonomous parvovirus. More recently, a study reported the use of transcriptional targeting in redirecting autonomous parvoviral replication to colon cancer cells. In most colon tumors the wnt-signalling pathway is constitutively activated due to mutations in the adenomatous polyposis coli and β -catenin genes, leading to expression of genes involved in cell activation and cell cycle. Insertion of binding sites for the heterodimeric β -catenin/Tcf transcription factor into the parvoviral P4 promoter, resulted in wild-type replication kinetics in colon cells with activated wnt-signalling pathways, while burst size was reduced at least 1000-fold in cell lines with inactive wnt-signalling pathways.¹⁰³

Ways to enhance viral antitumor activity

Enhancement of viral intrinsic antitumor potency

Although both many naturally occurring and engineered oncotrophic viruses have been demonstrated to display specific oncolytic activity, there are few examples of complete tumor eradication. Therefore, attempts have been made to enhance the cytolytic effect of replication competent viruses (see Table 4).

Expression of cytotoxicity enhancing proteins. Several classes of cytotoxic proteins are under evaluation for improvement of the intrinsic potency of oncolytic viruses to kill cells. One such protein is the nuclear membrane glycoprotein E3-11.6kd, termed adenovirus death protein (ADP), which mediates efficient cell lysis and release of progeny virus from cells.¹⁰⁴ However, ADP is deleted in the majority of oncolytic adenoviruses constructed to date. Adenovirus mutant CV787, containing a wild-type E3 region, showed enhanced target cell killing, relative to mutant adenovirus CV739 deleted for the E3 gene both *in vitro* and *in vivo*.⁸¹ Currently, CV787 is being evaluated in phase I and II clinical trials. Furthermore, Doronin et al¹⁰⁵ reintroduced the ADP gene back into the genome of E3-deleted, E1A-mutated replication competent viruses and

demonstrated that it was expressed at higher levels than in natural infection, and that these viruses were more efficient in cell lysis and in causing tumor regression than their E3-deleted counterparts. Alteration of expression of other adenoviral proteins can also improve viral cytotoxic potency. For example, an adenovirus deleted for the E1B-19kd protein, which is a potent inhibitor of apoptosis, displayed more efficient cell killing of several tumor cell lines by apoptosis than a wild-type virus.^{107,108} Furthermore, overexpression of viral protein E4orf4, which is known to induce apoptosis in infected cells independently of p53, might also enhance adenovirus mediated cell killing.¹⁰⁶ Expression of proteins that induce apoptosis have also been demonstrated to be beneficial to the intrinsic antitumor effect of autonomous parvovirus. A recombinant H1 parvovirus vector producing the apoptin protein (a tumor cell-specific, p53-independent, Bcl-2-insensitive apoptotic effector) exhibited pronounced increased cytotoxic tumor cell-specific potency compared to the wild-type H1 virus.³⁶

It has recently been shown that expression of the fusogenic membrane glycoproteins (FMGs) of measles virus and gibbon ape leukemia virus in oncolytic viruses led to cell killing by fusing them into large multinucleated syncytia. Treatment of U87 glioma xenografts in nude mice with adenoviruses, harboring F and H FMGs of measles virus, led to a significantly higher antitumor effect compared to a control adenovirus.¹⁰⁹ Similarly, an oncolytic HSV-1, in which genes of FMGs of gibbon ape leukemia virus were incorporated, showed a distinctive syncytial phenotype, leading to both increased cell killing ability *in vitro* and far better antitumor effects *in vivo*, relative to a nonfusogenic HSV-1.¹¹⁰ *In vivo* oncolytic potency of fusogenic HSV-1 was further enhanced when at the same time chemotherapeutic prodrug ganciclovir was administered.¹¹¹

Eliciting an antitumor immune response. Although most research concerning oncolytic virotherapy focuses on enhancement of direct virus-mediated tumor cell killing, there are also studies that evaluate the use of oncolytic viruses as tools to elicit primarily an antitumor immune response. One such approach uses human tumor cells infected with an oncolytic virus (so-called oncolysates) in order to enhance the antigenicity of the tumor tissue and, as a consequence, evoke a systemic immune response against tumor-specific antigens.¹¹² In recent phase III clinical trials of patients with stage III melanoma in a surgical adjuvant setting, administration of an vaccinia melanoma oncolysate, did not result in statistically significant improvement in disease-free interval or overall survival in all patients, but did confer a survival advantage in certain subsets of patients.¹¹³ In addition to vaccinia virus, NDV oncolysates have also been evaluated for eliciting melanoma-specific immune responses. Batliwalla et al¹¹⁴ performed a 15-year follow-up study of patients with stage III malignant melanoma treated with an NDV oncolysate as part of clinical phase II trial study, that revealed an overall 15-year survival of 55%. On the other hand, application of VSV in an

oncolysate as a treatment for melanoma was not effective.¹¹⁵

Furthermore, oncolytic viruses have been constructed that encode tumor-specific antigens in order to elicit an antitumoral immune response directly. Vaccinia viruses have been engineered that encode PSA or CEA, to generate an immune response against prostate cancer and several CEA expressing tumor types, respectively. A phase I trial showed that a PSA-encoding vaccinia virus was safe and, in combination with an immunostimulatory adjunct, it elicited PSA-specific T-cell responses and was able to inhibit tumor growth in several prostate cancer patients.¹¹⁶ In another phase I clinical trial of 18 patients with advanced tumors expressing (CEA), it was demonstrated that vaccination with vaccinia virus expressing CEA, induced T-cell responses and antibody production directed against CEA, when treatment was followed by administration of CEA gene harboring avipox virus. Further increases in CEA-specific T-cell precursors were seen when local granulocyte-macrophage colony-stimulating factor (GM-CSF) and low-dose interleukin (IL)-2 were given with subsequent vaccinations.¹¹⁷

Direct transfer of cytokine genes to tumor cells has emerged as a third immunotherapeutic tool in oncolytic virotherapy to increase its antitumor efficacy. The rationale behind this approach is that expression of cytokine genes in tumor cells, delivered by oncotropic viruses, will stimulate inflammatory and immune responses against these cells, thereby enhancing the antitumor effects of oncolytic viruses. Zhang et al¹¹⁸ developed a replication competent adenoviral vector, in which the human interferon consensus gene was inserted, and showed that treatment of human breast cancer xenografts with this virus was associated with stronger tumor regression relative to the wild-type virus. Moreover, several oncolytic HSV-1s have been constructed that encode immunostimulatory agents. In several mouse models, it was shown that treatment with HSV-1 into which either IL-4¹¹⁹ or IL-12^{120,121} was incorporated, resulted in a significantly prolonged survival and stronger reduction in tumor volume compared to wild-type virus. Importantly, treatment was generally accompanied by accumulation of inflammatory cells, like CD4⁺ and CD8⁺ T-cells, suggesting that the increased therapeutic effect was caused by the induction of an antitumor immune response. Indeed, upon subsequent rechallenge with tumor cells, only 43% of the mice treated with HSV-1-expressing IL-12 developed tumors compared to 86% of the mice treated with an analogous noncytokine-carrying HSV-1.¹²¹ Furthermore, in a phase I clinical trial with patients with surgically incurable subcutaneous melanoma, i.e. injections of a vaccinia virus carrying the gene for GM-CSF resulted in leukocytes infiltrates at the inoculated sites and five out of seven patients had complete or partial responses.¹²² On the other hand, treatment of patients bearing treatment-resistant malignant mesothelioma with vaccinia virus-IL-2 induced only a T-cell infiltrate in 50% of the tumor biopsies and no tumor regression was observed.¹²³ The use of viruses with intrinsic oncotropic properties, like autonomous

parvovirus, have also been evaluated as vectors to efficiently express therapeutic cytokines in human tumor cells. An autonomous parvovirus, H1, expressing IL-2 displayed better oncolytic properties than wild-type virus, as the formation of tumors from HeLa xenografts was reduced by 90% compared with wild-type or mock-infected cells probably due to infiltration of natural killer cells into tumor tissue.³⁷

Altogether these studies demonstrate that expression of cytotoxicity-enhancing proteins and/or elicitation of an antitumor immune response are generally beneficial the antineoplastic effect of oncolytic viruses, and provide support for the use of these strategies in a therapeutic setting.

Oncolytic virotherapy in a multimodal antitumor approach

On the other hand, to increase the antitumor effect of oncolytic virotherapy, a multimodal cancer therapy is under evaluation utilizing oncolytic viruses in combination with chemotherapy, radiotherapy, or suicide gene therapy (Table 5). The rationale for this combination strategy is that combining therapies may have greater antitumor effects than either of these therapies alone. Second, the therapies have different safety profiles and may therefore without increased adverse events result in enhanced efficacy. Third, no overlapping resistance between oncolytic viruses and the other therapies is expected. And finally, it may be possible to lower treatment doses, thereby decreasing possible unwanted toxicity and still achieve greater efficacy than with single therapies.

Oncolytic viruses combined with chemotherapy. The antitumor activity of several oncolytic viruses in combination with chemotherapeutic drugs has been examined. Combined treatment of engineered adenoviruses CV787 and CV890, both targeted to PSA⁺ prostate cells, with doxorubicin¹²⁴ (a topoisomerase II inhibitor that blocks DNA synthesis) or paclitaxel and docetaxel (taxane antimicrotubule agents)¹²⁵ resulted in synergistic cytotoxic effects both *in vitro* and *in vivo*. Similarly, the antitumor effects of ONYX-015 in combination with chemotherapeutic drugs 5-fluorouracil (a pyrimidine antagonist that inhibits DNA synthesis),¹²⁶ cisplatin (causing intrastrand DNA crosslinks that block DNA replication),¹²⁷ doxorubicin or paclitaxel^{128,129} were strongly enhanced or synergistic in several human tumor models (see Table 5). Combination therapy of ONYX-015 and chemotherapeutic drugs in clinical trials demonstrated the safeness of this treatment modality.^{55,56,130,131} However, in only one clinical trial significant higher tumor regression rates were evidenced compared to either monotherapy alone¹³⁰ (see Table 6). Furthermore, some mouse model studies have reported strongly enhanced oncolytic efficiency of engineered HSV-1 vector G207 when applied in combination with cisplatin¹³² and vincristine.¹³³ These studies show that the therapeutic effect of oncolytic viruses combined with chemotherapy is

enhanced compared to the effect of either therapy alone and this dual mode of therapy can even exert synergistic effects in some instances. Importantly, chemotherapy did not inhibit the cytotoxic properties of the oncolytic viruses and, in some cases, lower treatment doses were sufficient to still exert increased antitumor effects compared to treatment with either therapy alone.

Oncolytic viruses combined with radiotherapy. The additive tumor-killing effects of several adenoviral vectors in combination with radiotherapy have been evaluated. Rogulski et al⁴² demonstrated that ONYX-015 viral therapy when combined with radiotherapy improved tumor control beyond that of either monotherapy. The therapeutic effect of another adenoviral vector, Ad5-Δ24RGD, which contains an E1A mutation and an RGD-motif insertion in its fiber, resulting in retargeting to α_v integrins expressing cells with pRb-pathway defects, in combination with radiotherapy was strongly enhanced in glioma cells both *in vitro* and *in vivo*. Furthermore, the cytotoxic effects of CV706, a prostate cell-specific adenovirus variant, toward the human prostate cancer cell line LNCaP was demonstrated to be synergistically enhanced when used in combination with radiation with no reduction in specificity.¹³⁴ Moreover, the safeness of CV706 was confirmed in a phase I dose escalation study with patients with locally recurrent prostate cancer.⁸⁰ Enhanced therapeutic effects were also observed when treatment of an HSV-1 mutant NV1020, lacking both copies of the γ 34.5 gene, was combined with ionizing radiation both in human U-87 malignant glioma^{135,136} and Hep3B hepatoma¹³⁷ xenografts-bearing mice. On the other hand, combination therapy of low-dose radiation and infection with another replication-selective HSV mutant, G207, did exhibit increased antitumor effects against cervical tumors *in vitro* and *in vivo*¹³⁸ but not in a prostate tumor mouse model.¹³⁹

Altogether, these preclinical studies have shown us the beneficial effects of radiotherapy to oncolytic virotherapy. Several findings from these studies warrant further investigation. First, radiation did not impair viral replication, and, in several cases it even increased viral replication titers.^{135,137} Thus, this indicates that, unlike human genomes, viral genomes suffer relatively little damage by ionizing radiation, enabling them to replicate still with similar or even enhanced efficiency. Second, these studies have demonstrated that toxicity was not significantly increased in dual treatment regimens, compared to single therapies. Lastly, combination therapy of irradiation with nearly all tested oncolytic viruses led to an enhanced and sometimes even synergistic antitumor effect, relative to monotherapies. Although these preclinical studies are quite promising, clinical trials will have to be initiated to determine whether this mode of therapy is safe and can be used as a more efficient antitumor approach in patients as well.

Oncolytic viruses combined with suicide gene therapy. A third dual therapy approach that has been examined for enhanced antitumor efficacy of oncolytic viruses is the

Table 5 Oncolytic virotherapy in combination with standard therapies in preclinical studies

Parental strain	Agent	Chemotherapeutic drug	Effect	Tumor type	References
<i>Chemotherapy treatment</i>					
Adenovirus	CV890	Doxorubicin	Synergistic	Liver cancer	124
		Paclitaxel	Synergistic	Prostate cancer	125
		Docetaxel	Synergistic	Prostate cancer	125
	ONYX-015	Doxorubicin	Synergistic	Thyroid cancer	128
		Paclitaxel	Synergistic	Thyroid cancer	128
		5-Fluorouracil	Enhanced	HNSCC, colon cancer	126
		Cisplatin	Enhanced	HNSCC	126
		Cisplatin+paclitaxel	Synergistic	NSCLC	129
		Cisplatin+5-fluorouracil	Enhanced	HNSCC, ovarian cancer	127
		Vincristine	Enhanced	Rhabdomyosarcoma	133
		Cisplatin	Synergistic	HNSCC	132
HSV-1	G207				
<i>Radiotherapy treatment</i>					
Adenovirus	ONYX-015		Additive	Colon cancer	42
	Ad5-Δ24RGD		Enhanced	Glioma	164
	CV706		Synergistic	Prostate cancer	134
HSV-1	NV1020		Synergistic	Glioma	135,136
	G207		Enhanced	Cervical cancer	138
			No effect	Prostate cancer	139
<i>Suicide gene therapy treatment</i>					
Adenovirus	Ad-TK	Thymidine kinase	Enhanced	Glioma	140
			Enhanced	Colon cancer	141
			No effect	Colon cancer	143
			No effect	Intraperitoneal tumours	144
			No effect	HNSCC	142
	Ad5-CD/TKrep	Cytosine deaminase/thymidine kinase fusion	Enhanced	Prostate cancer, glioma	148
			Enhanced	Cervical cancer	149
			Enhanced	Colon adenocarcinoma	165
			Enhanced	Hepatocellular carcinoma	152,153
			Enhanced	Glioma	154
Vaccinia virus	VVCD	Cytosine deaminase	Enhanced	Glioma	147
HSV-1	rRp450	Cytochrome P450 oxidase	Enhanced	Mammary carcinoma	
VSV	VSV-C:U	Cytosine deaminase	Enhanced		
<i>Suicide gene therapy+radiotherapy treatment</i>					
Adenovirus	Ad5-CD/TKrep	Cytosine deaminase/thymidine kinase fusion	Enhanced	Prostate, glioma	148
			Enhanced	Cervical carcinoma	149
Adeno-associated virus	AAVtk	Thymidine kinase	Enhanced	Laryngeal cancer	156

HSV-1, herpes simplex virus-1; VSV, vesicular stomatitis virus; HNSCC, head and neck squamous cell carcinoma; HSCLC, human small-cell lung carcinoma.

combination of oncolytic viruses with so-called suicide gene therapy. This strategy uses replication conditional viruses as gene transfer vehicles to specifically deliver 'suicide' genes to tumor cells. These suicide genes encode enzymes that convert an exogenously administered non-cytotoxic prodrug into a noxious drug. By this mechanism, ideally, only tumor cells are killed due to disruption of DNA replication by the converted drug, while nontransfected cells remain unharmed. Thus, with this approach, systemic toxicity commonly associated with conventional chemotherapy is minimized. Of the many enzyme genes that have been studied for more efficient tumor cell killing by replication defective viruses, three are

currently being evaluated in the context of replication competent viruses.

First, the most extensively studied prodrug-suicide gene combination is composed of the antiviral prodrug ganciclovir (GCV) and the HSV thymidine kinase gene (HSV-tk). The viral tk enzyme monophosphorylates GCV, which is then converted by cellular kinases to GCV triphosphate, an inhibitor of viral and cellular DNA synthesis. In addition, activated GCV can diffuse to neighboring, uninfected cells through gap junctions leading to additional cell killing above the direct cytotoxic effects of oncolytic viruses (Fig 3). Introduction of the HSV-*tk* gene into oncolytic adenoviruses led to enhanced

Table 6 Examples of oncolytic viruses in clinical trials

Parental strain	Agent	Genetic alteration	Cancer targeted	Combined with other therapy	Phase	Reference	Therapeutic effect
Adenovirus 2/5 chimera	ONYX-015	E1B-55kd deletion	SCCHN	No	II	49	14% pr to cr, 41% sd, 45% pd
				No	I	51	No objective responses
				Chemotherapy	II	130	27% cr, 36% pr, 37% sd
				No	I	53	No objective responses
			Ovarian cancer Primary and secondary liver tumors	No	I/II	54	No objective responses
				Chemotherapy	II	56	26% pr, 33% sd, 41% pd
			Pancreatic cancer Colorectal cancer	No	I	52	No objective responses
				Chemotherapy	I/II	131	19% pr, 29% sd, 52% pd
			Prostate cancer	No	II	58	22% sd, 78% pd
				Suicide gene therapy Suicide gene therapy+ Radiotherapy	I	150	25% PSA decline in 63% of patients
Adenovirus (serotype 5)	Ad5-CD/TKrep	E1B-55kd deletion; Insertion of HSV-tk/CD fusion gene	Prostate cancer	Suicide gene therapy Suicide gene therapy+ Radiotherapy	I	155	Significant PSA decline in all patients
	CV706	Regulation of E1A under PSA promoter; E3 deletion	Prostate cancer (organ confined)	Radiotherapy	I	80	Significant PSA decline in all patients
	CV787	Regulation of E1A under rat probasin promoter and E1B under PSA promoter; wild-type E3	Prostate cancer (organ confined and metastatic)	No	I	Underway	—
Herpes simplex virus-1	G207	Deletion of both α 34.5 genes; ribonucleotide reductase disruption	Malignant glioma	No	I–II	71	No replication data available, but probably responses in some patients
	NV1020	Deletion of one γ 34.5 gene; deletion in tk gene; insertion of exogenous copy of tk gene	Colorectal carcinoma liver metastases	No	I	Underway	—
Vaccinia virus	Vaccinia-oncolysate	None	Melanoma	No	III	113	Survival advantage in subset of patients
	Vaccinia-GM-CSF	Insertion of GM-CSF gene into tk gene	Melanoma	No	I	122	14% cr, 57% pr to mr, 29% pd, tumor-specific T cells
	Vaccinia-CEA	Insertion of CEA gene	CEA expressing tumors	No	I	117	CEA-specific T cells and antibodies
	Vaccinia-PSA	Insertion of PSA gene	Prostate cancer	No	I	116 166	42% sd, PSA specific T cells PSA specific T cells
Newcastle disease virus	73-T-oncolysate	Naturally attenuated	Melanoma	No	II	114	Overall 15-year survival of 55%, tumor-specific T cell response
	PV701	Naturally attenuated	Advanced solid cancers	No	I	28	4–31 months sd, tumour specific T cell response
	MTH-68/H	Naturally attenuated	Glioblastoma	No	I	167	5–9 years survival rates
Reovirus	Reolysin	None	SCCHN	No	I	168	?

SCCHN, squamous cell carcinoma of head and neck; tk, thymidine kinase; PSA, prostate-specific antigen; CD, cytosine deaminase; cr, complete response; pr, partial response; sd, stable disease; pd, progressive disease.

antitumor effects when these viruses were administered in conjunction with GCV prodrug, compared to treatment with the virus alone.^{140,141} In contrast, other studies have shown that addition of GCV did not lead to an increase in antitumor effect of HSV-tk-carrying adenoviruses.^{142–144} This might be explained by the fact that GCV, like the other prodrugs used in these studies, interferes with DNA synthesis in general, thereby being able to attenuate viral replication as well. This could lead to impairment of the oncolytic effect, resulting in an antagonistic action.

Second, *Escherichia coli* cytosine deaminase gene (CD) has also been evaluated for enhancement of therapeutic efficacy of oncolytic viruses. The CD enzyme converts prodrug 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU), which blocks DNA synthesis. In this context, a replicating vaccinia virus expressing the CD gene showed improved antitumor effects *in vitro* and *in vivo*, compared with virotherapy alone. The effect of the CD/5-FC suicide gene mechanism on the oncolytic potency of autonomous parvovirus (H1) has also proven to be beneficial, particular in combination with IL-2.¹⁴⁵ Similar results were obtained from studies of an E1B⁻ adenovirus harboring a ZD55-CD gene¹⁴⁶ and a recombinant VSV in which the CD/uracil phosphoribosyltransferase gene was inserted.¹⁴⁷ Freytag et al¹⁴⁸ went one step further by evaluating the concept of combining two suicide gene systems with oncolytic virotherapy. The resulting E1B⁻ adenoviral vector, termed Ad5-CD/TKrep, containing a fusion gene encoding both cytosine deaminase (CD) and HSV-tk, exhibited markedly enhanced tumor cell-specific cytopathic effects in combination with either 5-FC or GCV administration, with the greatest effect when the virus was administered in combination with both prodrugs.^{148,149} In addition, in a phase I study with recurrent prostate cancer patients Ad5-CD/TKrep treatment did not only appear to be safe, but also resulted in an objective decrease in PSA levels.¹⁵⁰

A third suicide gene that has been studied in combination with oncolytic viruses is the CYP2B1 gene. It encodes the cytochrome P450 oxidase responsible for converting prodrugs like CPA to its DNA synthesis inhibiting metabolite phosphoramidate mustard, which also diffuses to neighboring cells to produce bystander killing. A replication competent HSV-1 mutant, designated rRp450, has been constructed containing the CYP2B1 gene within its ribonucleotide reductase locus¹⁵¹ (see Figure 3). rRp450-mediated oncolysis in the presence of CPA was significantly increased both *in vitro* and *in vivo*.^{152,153} In contrast, as the viral *tk* gene is still intact, cytotoxicity was reduced in the presence of ganciclovir^{152,153} although others have found the opposite¹⁵⁴ (a possible explanation for these contradicting data has been described above).

Since the prodrug-suicide gene systems described here all exhibit potential antiviral activity, it is clear that timing and dose of prodrug delivery will be critical in the outcome of how viral replication is affected and will ultimately determine the antitumor efficacy of the combined therapy. Evaluation of other suicide gene systems will be important in order to find a system that

does not attenuate viral replication and primarily possesses anticancer properties.

Oncolytic viruses in conjunction with both suicide gene therapy and radiotherapy. Efforts have been made to further enhance the cytopathic potency of oncolytic viruses by combining oncolytic virotherapy with both suicide gene therapy and radiotherapy. It was found that a trimodal approach involving replication competent adenovirus, Ad5-CD/TKrep, containing both CD/5-FC and tk/GCV suicide gene systems, double suicide gene prodrugs, and radiotherapy led to greater cytotoxic effects compared to other dual treatment combinations.^{148,149} Recently, in a phase I clinical trial in patients with prostate cancer, the safety and efficacy of this three-pronged therapy was demonstrated,¹⁵⁵ with significant declines in PSA levels in all treated patients. A recent study reported that the trimodal approach was also very effective in potentiating the antitumoral activity of adeno-associated virus vectors.¹⁵⁶ Taken together, it is anticipated that Ad5-CD/TKrep and possibly other vectors as well, in combination with suicide gene and radiation therapy, will have greater potential of destroying tumor cells and improving the survival of cancer patients.

Discussion

Limitations and potential hurdles to overcome

Although the oncolytic potency of both unmodified and genetically engineered viruses has been demonstrated in preclinical studies, the use of oncolytic viruses in destroying tumors in clinical trials has in most cases not yet been very successful (an overview of the oncolytic viruses that are currently evaluated in clinical trials is given in Table 6). Several factors have been identified that might explain this little therapeutic effect of oncolytic virotherapy in cancer patients in current studies.

In the first place, immune responses are likely to play a critical role in the antitumor efficacy of the therapy. On the one hand, it has been demonstrated that in addition to direct cytotoxic effects of viruses, viral treatment can elicit an antitumor immune response, that is beneficial to the outcome of the therapy.^{157–159} This is why an important strategy to enhance the antitumor potency of oncolytic viruses is based on this phenomenon. On the other hand, however, there are several reports indicating that immune responses can also lead to impediment of the antitumor activity of virotherapy particularly during systemic treatment modalities.^{22,160} Interestingly, the presence of high neutralizing antibodies titers does not abrogate viral infection and replication following i.t. injection.⁵⁰ Probably, the immune system antagonizes the effectiveness of oncolytic viruses administered systemically, through inactivation of the virus, whereas once the virus has reached the tumor or is injected directly into it, the immune system will not negatively affect viral treatment anymore and might in theory enhance tumor regression, as is illustrated by studies summarized in Table 4.

Furthermore, efficient viral spread within a tumor has been recognized as one of the most important parameters for antitumor efficacy following i.t. or systemic treatment. However, viral spread in solid tumors *in vivo* is often limited.¹⁶¹ Possibly, ineffective viral spread might relate to the relative large sizes of the viruses (for example, 90 and 120 nm for adenovirus and HSV-1, respectively), mixture of the tumor cell mass with normal cells (up to half of the cells in some tumors) or low expression of receptors needed for viral infection. It will be important to further evaluate approaches that improve viral spread within a tumor.

Lastly, there are indications that some viruses, for example ONYX-015, demonstrate less cell killing potential in a clinical setting than was expected from preclinical studies.^{41,42} Another factor that could potentially limit efficient tumor cell killing by oncolytic viruses in cancer patients is the heterogeneity of tumor cell populations. As a result, it is likely that oncolytic viruses are able to kill some tumor cell populations more efficiently than others.

Safety concerns

Apart from the therapeutic potency, the feasibility of oncolytic virotherapy will also depend on its safety profile. In the first place, the pathology and toxicity profile of oncolytic viruses should be determined. Until today, the safety of a small selection of engineered oncotropic viruses has been demonstrated in clinical trials (e.g. HSV-1 and adenovirus-derived vectors). Second, it will be important to determine the tropism of a newly genetically engineered virus. For instance, narrowing the tropism of a virus would pose no serious risk. Conversely, it could be dangerous when the tropism of a virus is extended to other tissues. Third, it should be examined what the chances and consequences are of reversion to or recombination with wild-type strains. Lastly, the ability to be transmitted to other humans could also pose a threat. In general, insertion of prodrug-activating enzyme genes, like the HSV-tk gene, will provide a safety mechanism that shuts down viral replication should nonspecific pathology arise or transmission occur.

Conclusions

The idea of using viruses to destroy tumor cells is tantalizing for the simplicity of its principle, and for the perspectives it raises. Unfortunately, their effective application is not as straight forward as we would have hoped for. Although, preclinical studies are quite promising, data from clinical trials are far less convincing. Although the overall safety of viral treatment was demonstrated, in most cases no objective antitumor responses were evidenced. These clinical trials have pointed out that hurdles like immune responses, systemic distribution and intratumoral spread are major limitations of this mode of therapy. In addition, they have highlighted the weakness of extrapolating therapeutic efficacy observed in preclinical studies to cancer patients.

To overcome these hurdles, it will still be of great importance to further optimize existing strategies and evaluate new possibilities in order to optimize the antitumor efficacy of oncolytic virotherapy, by for example combining multiple tumor-targeting mechanisms (manipulation of viral attachment proteins, the use of tumor-specific promoters and/or viral gene deletions) within one virus. On the other hand, combination of these tumor selectivity improving strategies with several toxicity-enhancing genes, such as genes encoding cytotoxic proteins, prodrug-converting enzymes and cytokines, could further potentiate the oncolytic potential of a virus and enable better viral spread within a tumor. Especially, the ability to elicit an antitumor immune response will be of great importance, because it is likely to be critical to the long-term success of the treatment. However, it is unknown whether all these features can be combined in one virus while maintaining replicative viability. Moreover, it raises further safety concerns due to altered phenotypic characteristics. Therefore, possession of a 'fail-safe' mechanism to prevent unwanted viral spread would be essential. Lastly, the therapeutic success will, for a large part, be dependent on the pharmacokinetic characteristics of administered viral vectors in patients. It will therefore be very important to get a better insight in the factors, like antiviral immune responses, that affect the pharmacology of oncolytic viruses. In this respect, optimization of the route of viral administration and the use of viruses that exhibit low immunogenicity and do not elicit a strong antiviral immune response could contribute to a better delivery to tumors and thereby higher therapeutic efficacy.

Altogether, despite the fact that additional modification of oncolytic viruses might result in increased oncolytic potency, as has been demonstrated with several second generation viruses, it is still unlikely that oncolytic virotherapy as a monotherapy will be able to fully eradicate tumors in a clinical setting. Therefore, as both preclinical and clinical trials suggest, inclusion of oncolytic viruses into a multimodal cancer treatment approach composed of standard radio-, gene-, and chemotherapies, will have the greatest potential of destroying tumors and improving the survival of cancer patients. It can be anticipated that further evaluation of oncolytic viruses as therapeutic tools in this setting will contribute to the extension of our anticancer armamentarium in the future.

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