J Mol Med (2002) 80:163–175
DOI 10.1007/s00109-001-0295-1

REVIEW

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Replication-selective viruses for cancer therapy

Received: 7 May 2001 / Accepted: 1 October 2001 / Published online: 20 December 2001

Abstract Advances in our understanding of the molecular basis of cancer and the availability of technology to genetically engineer viruses have led to the development of replication-competent viruses to treat cancer. In theory, replication-selective viruses offer several appealing properties as biological agents for cancer therapy: they kill tumor cells selectively, and their replication leads to amplification of their oncolytic potential. Most preclinical experiments in tissue culture and in animal models support this notion. Clinical data on the first generation of replication-selective viruses are now rapidly accruing. The therapeutic index, and ultimately the clinical outcome, will depend on a complex balance between host and viral factors. This review discusses strategies to kill cancer cells based on our understanding of their molecular defects and the progress being made using replication-competent viruses for tumor therapy. We focus our discussion on a replication-selective adenovirus called ONYX-015 that has recently demonstrated encouraging results in clinical trials

Keywords Replication-selective viruses · Cancer therapy · Tumor; clinical trials · ONYX-015

Abbreviations *5-FU:* 5-Fluoruracil · *ARF:* Alternative reading frame · *HPV:* Human papilloma viruses · *HSV:* Herpes simplex virus · *IL:* Interleukin · *NDV:* Newcastle disease virus · *PSA:* Prostate-specific antigen · *Rb:* Retinoblastoma

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Introduction

Although standard treatment modalities for cancer such as surgery, chemotherapy, and radiotherapy have improved over the past decades, most neoplasms remain incurable. Novel therapeutic approaches are required which are based on our current understanding of the mo-

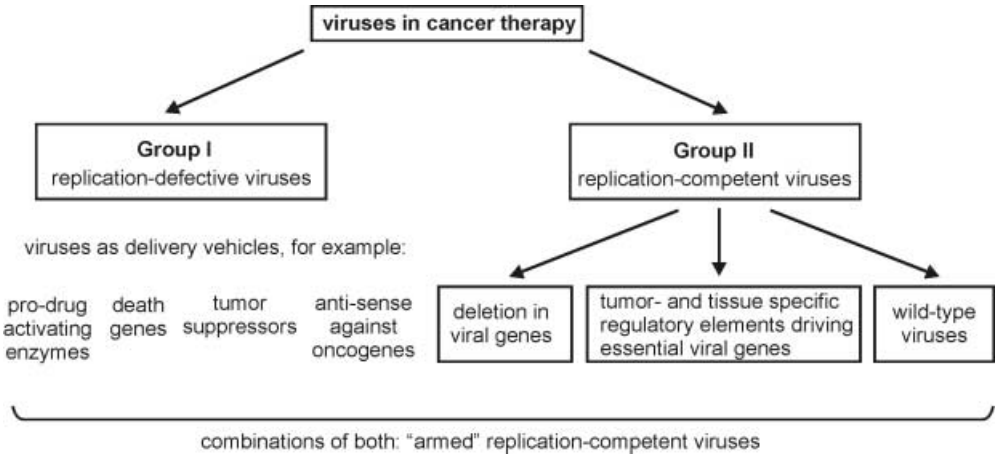


Fig. 1 Strategies for viral cancer therapies. Viruses can be used as delivery vehicles for different types of therapeutic molecules (group I). Alternatively, viruses can be genetically modified to achieve tumor-selective replication and tumor lysis (group II)

lecular basis and genetic etiology of cancer. Several types of cancer gene therapy strategies have been developed. Many of these depend on the delivery of a toxic drug or death gene to the tumor cells using viral (e.g., adenoviruses, retroviruses, adenoassociated viruses) or nonviral (e.g., liposomes, naked DNA injection) delivery systems (group I, Fig. 1). Others involve replacement of wild-type tumor suppressor genes such as p53 in tumor cells that have lost its expression due to deletion or mutation [1]. A third approach is based on methods to diminish the production of an oncogene (e.g., Mdm2) whose expression is aberrantly regulated in tumors [2, 3]. Replication-defective viral vectors have been used to allow efficient delivery of a variety of transgenes to target tissues [4, 5, 6]. However, because these gene delivery vectors cannot discriminate between tumor cells and normal cells they may mainly be useful in local/regional therapy. A means to circumvent this issue would be to design a vector that amplifies itself via replication in tumor cells, but not in normal cells. Replication-competent viruses have several appealing properties as biotherapeutic agents. While conventional chemotherapy follows log cell kill kinetics, in which a proportional number of cells are killed with a given dose of drug regardless of the tumor burden, replication of the oncolytic virus in tumors in theory amplifies the input dose and helps spread the agent to adjacent tumor cells. This increases tumor transduction efficiency, creates a high local concentration of tumoricidal virus, and thus augments therapeutic efficacy. Utilization of viruses which replicate in tumor cells through a lytic cycle will result in oncolysis. Furthermore, additional mechanisms have been reported to explain virus-induced tumor regression. Viral proteins associated with either tumor cell membrane fragments or intact cells may enhance the immunogenicity of tumor antigens, thereby augmenting host cellular- and humoral-mediated destruction of the tumor [7, 8, 9]. Certain viruses may stimulate host production of cytokines, for example, interferon and tumor necrosis factor-α [10, 11]. Although for nearly half a century the idea of using replication-competent viruses to treat human cancer has been revisited, only recently has our understanding of viral and cancer biology progressed to allow genetic engineering of replication-selective tumor-specific viruses. In this review we discuss strategies to kill cancer cells based on our understanding of their molecular defects and the progress being made using replication-competent viruses for tumor therapy. We focus on a replication-selective adenovirus called ONYX-015 that has recently demonstrated very encouraging results in clinical trials.

Types of replication-competent viruses used for cancer therapy

Several strategies have been applied to achieve tumor selective replication of viruses (group II, Fig. 1). A first concept is to delete viral genes essential for replication in normal (primary) cells but not in tumor cells. A second approach is to use genetically modified promoters to control the expression of essential viral genes through binding of tissue- or tumor-specific transcription factors. Third, suicide genes can be delivered by viruses that replicate selectively in tumor cells utilizing one of the strategies mentioned above and thereby enhance the tumorlysis. Finally, for some viruses [e.g., reovirus, Newcastle disease virus (NDV)] a natural selectivity of replication in tumor cells has been reported [11, 12, 13].

Genetic alterations are the basis for tumor development

Alterations in the cellular genome affecting the expression or function of genes controlling cell growth and differentiation are considered to be the main cause of cancer [14]. It is now evident that tumorigenesis is a multistep process in which mutations targeting tumor suppressors and proto-oncogenes accumulate [15, 16]. Although we do not fully understand all steps required for cellular transformation, alterations in a number of signaling pathways seem to be a common theme in cancer development. For example, most if not all tumors have defects in the p53 pathway, either in p53 itself or in proteins that

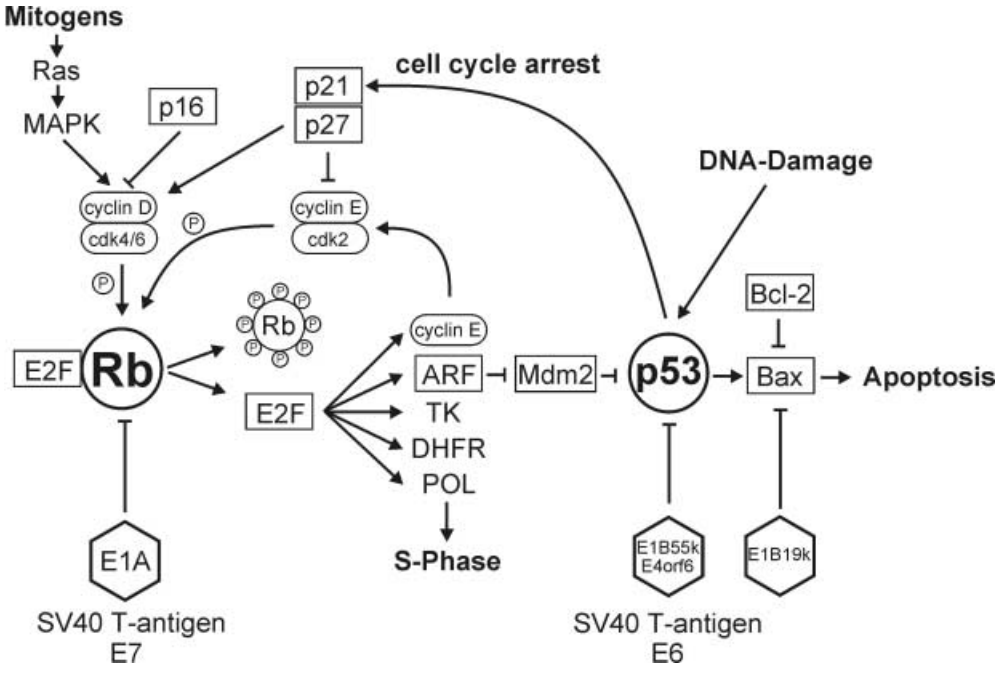


Fig. 2 Interaction between cellular and viral factors in the regulation of the cell cycle. Mitogens such as growth factors activate the Ras/Raf/mitogen-activated protein kinase (*MAPK*) cascade, which leads to phosphorylation of the retinoblastoma protein (*Rb*) via activated cyclinD–cdk4/6 complexes. E2F is released from Rb and drives the expression of several genes essential for S-phase entry of the cell cycle. p53, which can be induced either by DNA damage or via the alternative reading frame (*ARF*) pathway, is a transcription factor that drives the expression of the cell cycle inhibitor p21 and the apoptosis inducer Bax after cellular stress. The cell cycle is either arrested until the DNA damage is repaired or, if the damage is not repairable, the cell undergoes apoptosis. Viruses have evolved to produce a series of proteins that mimic cellular regulators of the cell cycle. The adenoviral E1A protein can bind to Rb and release E2F, driving the cell cycle into S phase. The SV40 T antigen and the human papilloma viruses (*HPV*) E7 protein have similar functions. Furthermore, E1B55K and E4orf6, both adenoviral proteins, are able to bind to p53 and inactivate it. Inactivation of p53 is also achieved by the SV40 T antigen and the HPV E6 proteins. Finally, the adenoviral E1B19K protein, a viral homologue of Bcl-2, can inhibit the function of Bax thereby blocking apoptosis

regulate it, Mdm2 and $p14^{ARF}$ [17, 18, 19]. Likewise, many tumors have defects in a pathway that includes cyclin D1, cdk4, p16INK4a, and the retinoblastoma (Rb) protein [20] (see Fig. 2).

During the G_1 phase of the cell cycle the Rb protein in its hypophosphorylated form binds to transcription factors of the E2F family, keeping them in an inactive state [21]. When normal primary cells are exposed to mitogenic signals, such as growth factors, the cyclin D dependent kinases cdk4 and cdk6 are activated and trigger Rb phosphorylation in the middle to late G_1 phase [22, 23]. E2F is released and subsequently activates a series of target genes whose expression is required for cells to progress into S phase of the cell cycle, thereby stimulating proliferation [24]. The p53 protein is a transcription factor that can block cell cycle progression or induce apoptosis in response to stress or DNA damage [25, 26]. In its role as a tumor suppressor p53 serves as the "guardian of the genome" by regulating critical checkpoints in response to the distinct stresses. p53 levels and activity increase following DNA damage, owing in part to de novo phosphorylation and conformational changes [27, 28]. Phosphorylation at specific serine residues by protein kinases, such as DNA-PK and ATM, prevent the interaction of p53 with Mdm2, a protein that can downregulate p53 via ubiquitin-mediated proteolysis [29, 30]. p53 accumulates in the nucleus of the cells and activates transcription of a variety of genes, such as $p21^{WAF1}$ or Bax, thereby inducing growth arrest and apoptosis [31, 32] (see Fig. 2).

Uncontrolled activation of E2F that results from the loss of Rb function contributes to uncontrolled entry into S phase of the cell cycle [20]. However, hyperactivation of E2F leads also to increased expression of a protein named $p14^{ARF}$ [33, 34]. $p14^{ARF}$ binds directly to Mdm2, sequestering it into the nucleolus and enabling transcriptionally active p53 to accumulate in the nucleoplasm [35, 36]. Furthermore, $p14^{ARF}$ inhibits the Mdm2 ubiquitin ligase activity, preventing Mdm2 from targeting p53 for degradation [37]. Interestingly, the *INK4a* gene locus encoding $p14^{ARF}$ also encodes the cyclin-dependent kinase inhibitor *p16Ink4a*, which is a component of the Rb pathway and negatively regulates the activity of the cyclin D/cdk4 complexes [38]. The mRNAs for the two tumor suppressors share common exons 2 and 3, but differ in exon 1 (designated 1α for p16Ink4a and 1β for $p14^{ARF}$). Since the initiator codon in exon 1β is not in frame with sequences encoding p16Ink4a in exon 2, the two gene products share no protein homology and the β-transcript is therefore designated $p14^{ARF}$ ("alternative reading frame") [39].

In human cancers disruption of the Rb pathway can result from inactivation of Rb itself through gene muta-

tion, deletion of the gene locus, or disregulation of the components controlling the degree of Rb phosphorylation [40, 41]. The latter can take place through activating mutations in the cdk4 catalytic unit, up-regulation of D type cyclin levels, or elimination of inhibitors for cdk4, such as p16Ink4a [20]. These pathogenetic events appear to be exceeded in frequency only by p53 inactivation. Mutations in p53 itself occur in about 60% of human cancers [42]. The DNA-binding central domain of p53 is the primary target for mutational inactivation in many cancers, and the most common mutation hotspots are involved in direct contact with the p53 DNA-binding site [43]. Not only p53 itself but also other components of the p53 pathway are targeted during cellular transformation to allow the bypassing of the p53-dependent cell-cycle checkpoint [44, 45, 46, 47]. Amplification of the *Mdm2* gene locus or deletion of $p14^{ARF}$ have been described as alternative mechanisms of inactivating the p53-dependent checkpoint. Defects in the p53 pathway, then, allow tumor cells to exert the positive effects of E2F, cell growth, and proliferation. Although mutation of p53, amplification of *Mdm2*, or loss of $p14^{ARF}$ seem to be equivalent in inactivating the p53 checkpoint, mutation in p53 itself has more severe consequences for tumor development and the clinical outcome. This is mainly because p53 is necessary for efficient cell killing by radiation or genotoxic chemicals [26]. Tumors that have lost $p14^{ARF}$ expression are still capable of responding to these insults activating p53 via multiple, distinct pathways.

Viruses and their interaction with cellular components

There are striking similarities between tumor cells and cells infected by viruses in their ability to interfere with signal transduction pathways promoting G_1 to S transition. In particular, p53- and Rb-dependent cell cycle checkpoints must be bypassed. Some viruses have evolved gene products that either interact physically with cell cycle regulatory proteins or transcriptionally activate their expression, thereby mimicking cell cycle activation in quiescent cells by physiological signals. As a representative example, the replication cycle of adenovirus 5 has been described, although it should be noted that many features bear similarity with SV40 and human papilloma viruses (HPV-16).

The replication cycle of adenoviruses has two phases, early and late, separated by the onset of DNA replication. A major function of the adenoviral early genes is to provoke the infected cell to enter the cell cycle and progress to S phase [48]. In S phase the virus can take advantage of the cellular DNA replication machinery and replicate its own genome efficiently. The viral E1A proteins are potent transactivators that relieve cellular growth suppression and induce quiescent cells to enter S phase by binding members of the Rb family, thereby releasing E2F [48]. Rb is also bound and inactivated by T antigen and the E7 protein, functional E1A homologues of the SV40 or HPV-16 viruses, respectively [49]. On the other hand, expression of E1A results also in induction of $p14^{ARF}$ and subsequent accumulation of active p53 in the nucleoplasm [50]. In response to viral infection p53 could induce G_1 growth arrest by inducing genes such as $p21^{WAF1}$ or apoptosis through the induction of Bax or other downstream mediators of apoptosis [31, 32]. Growth arrest or apoptosis early in infection would block viral replication and reduce viral yield significantly. Therefore adenoviruses encode another set of early genes, the *E1B* genes (*E1B55K* and *E1B19K*), that play a major role in protecting infected cells from these E1A-induced and p53-mediated effects [51, 52, 53]. The E1B19K protein is a functional homologue of the proto-oncogene Bcl-2 and prevents apoptosis by a similar mechanism [54, 55]. The large E1B protein complexes with the aminoterminal end of p53 and inhibits its activity as a transcription factor [51]. Furthermore, together with another early viral gene product, the E4orf6 protein, E1B55K exports p53 to the cytoplasm for degradation [56, 57]. In addition, at later times in the lytic infection the E1B55K protein in association with the E4orf6 protein facilitates the transport of viral late mRNA while inhibiting the transport of most cellular mRNA [58, 59]. It is now well documented that early proteins of a variety of DNA viruses inactivate the p53 function, thereby protecting from growth arrest and cell death after infection. For example, SV40 produces the large T antigen, which binds to and inactivates p53 and led to the discovery of p53 by coimmunopreciptiation in the late 1970s [60, 61]. HPV-16 produces E6 proteins that inhibit the transcriptional function of p53 [62]. Taken together, neutralization of p53 activity during infection seems to be essential for viral replication in the lytic infection.

Furthermore, there are a number of additional cellular pathways targeted in a cell type specific manner. Human T-lymphotropic virus 1 infection of human lymphocytes leads to constitutive activation of the Janus kinase, which contributes to T-cell immortalization by this virus [63]. The LMP-1 gene product produced by Epstein-Barr virus interferes with signaling by members of the tumor necrosis factor receptor family, contributing to transformation of human B-cells [64].

The use of an E1B55K-deleted virus (ONYX-015) to kill tumor cells

From the comparison between tumor cells and adenovirus-infected cells arose the following idea: Adenoviruses that are not able to block the p53 response should be defective for replication in normal cells but should grow efficiently in p53 mutated tumor cells. Since the majority of tumors have mutations in p53, these viruses should be useful for cancer therapy in a wide range of tumor patients. Adenoviruses are well characterized, and the functions of their early E1A and E1B proteins have been studied extensively. For this reason and because adenovi-

ruses are relatively easy to grow in culture, a mutant adenovirus, dl1520 that is deleted in the E1B55K gene, was chosen as a possible candidate [65]. This mutant is a human group C adenovirus that contains an 827-bp deletion in the E1B region and a point mutation that generates a stop codon preventing expression of a truncated form of the protein. It is incapable of degrading the p53 protein, which therefore accumulates in the nucleoplasm after infection of normal cells and may block viral replication. In theory, dl1520, which is now also referred to as ONYX-015, should replicate only in p53-deficient cells and therefore have the general property of being cancer specific.

The early E1A gene product of adenoviruses has been shown to be essential for viral replication. Segments of the E1A protein that bind to various cellular components such as Rb or p300 have been characterized [48]. However, it should be possible to construct viral mutants that lack critical regions of E1A, and might therefore replicate selectively in cells lacking functional Rb. These mutants are currently under evaluation and are discussed below.

Evaluation of ONYX-015 in cell culture

The ability of ONYX-015 to replicate in normal cells and tumor cell lines with known p53 status (wild-type or mutated/deleted) has been extensively studied [66, 67, 68, 69, 70, 71, 72]. In most cases replication of ONYX-015 in primary cells is attenuated 100- to 1000-fold compared to wild-type virus [67]. Initial results from 11 tumor cell lines suggested a correlation between p53 status and susceptibility to ONYX-015 [66]. However, further examination has established that no such correlation exists, because ONYX-015 can efficiently replicate in several cell lines despite their wild-type p53 status [68, 69, 70, 71]. Several reasons may account for these important findings, and are discussed below.

It was initially observed that ONYX-015 replicates in tumor cell lines with mutant or deleted p53 alleles. For example, in C33A cervical carcinoma cells, which express a p53 with an inactivating mutation at codon 273, ONYX-015 grows as efficiently as wild-type adenovirus [66, 67]. In many of these cancer cell lines, however, ONYX-015 grows more slowly than wild-type virus, but cells are killed in most cases producing high titers of the virus. This attenuation might be due to other functions of the E1B55K protein. As mentioned above, E1B55K has been demonstrated to modulate viral and cellular mRNA transport after transcription and processing but before translocating of mRNAs through the nuclear pores [58, 59]. It will be interesting to determine how tumor cells that support ONYX-015 replication efficiently compensate for the lack of the E1B55K export function. All these data support the original hypothesis that E1B55K should only be essential in cells retaining wild-type p53 function.

As stated above, several tumor cell lines have now been reported in which the p53 gene is wild-type and nevertheless allow efficient replication of ONYX-015 [68, 69, 70, 71]. We were interested to determine whether alternative mechanisms of inactivation of the p53 pathway can explain this finding. In a recent study we demonstrated that the lack of $p14^{ARF}$ expression disrupts the p53 pathway, thus facilitating replication of ONYX-015 in many tumor cell lines that retain a wild-type p53 allele [72]. As mentioned above, a role for $p14^{ARF}$ as a negative regulator of Mdm2, interfering with Mdm2-mediated shuttling and degradation of p53 has been shown previously [35]. Loss of $p14^{ARF}$ in tumor cells containing wild-type p53 causes a deregulation of Mdm2, thus inhibiting p53 from exerting its protective effects following infection. Reintroduction of $p14^{ARF}$ into those tumor cells inhibited replication of ONYX-015 by 0.5–1 log, but not wild-type adenovirus. Importantly, this protective effect of $p14^{ARF}$ is p53 dependent, as introduction of $p14^{ARF}$ into cells with deleted p53 alleles did not prevent ONYX-015 replication [72]. Collectively, the presence of two different types of tumors with wild-type p53 are suggested: type A, tumor cells with an intact $p14^{ARF}$/Mdm2/p53 pathway, which suppress replication of ONYX-015 in a p53-dependent manner; and type B, tumor cells with disrupted $p14^{ARF}$/Mdm2/p53 pathway that support replication of ONYX-015. This might be caused by deletion of $p14^{ARF}$ or amplification of the Mdm2 gene. It is important to mention that there are some tumor cell lines that inhibit replication of ONYX-015 in a p53-independent manner. For example, ONYX-015 fails to replicate in the osteosarcoma cell line U2OS [66, 71]. These cells retain wild-type p53 but express no $p14^{ARF}$. However, elimination of p53 function through expression of a dominant negative p53 did not support replication [71]. Clearly, in these cells another function of E1B55K is necessary. The identity of this function is unknown, but it can be speculated that it relates to the export of late viral mRNAs by E1B55K in infected cells.

Preclinical studies on ONYX-015 in mice

Studies in animal models are widely used to predict the efficacy and toxicity of a newly developed drug in vivo. The use of mouse knock-out models in which defined genes are deleted by homologous deletion could have contributed to the understanding of the cellular mechanisms underlying the replication of ONYX-015. However, a major problem is that human adenoviruses do not replicate efficiently in other species such as mouse. Experiments to generate a “mouse version” of ONYX-015, which is based on a mouse pathogenic adenovirus are currently under way.

To test whether ONYX-015 can indeed spread through the solid mass of a human tumor grown in nude mice C33A cervical carcinoma xenograft models were studied. Intratumoral injection of ONYX-015 resulted in significant tumor growth inhibition compared with control tumors (vehicle or UV-inactivated ONYX-015) [66]. More than 50% of C33A xenograft tumors completely

regressed. All responses have been lasting, without evidence of tumor regrowth after 6 months of posttreatment follow-up. The antitumoral effects of intratumoral or intravenous injections with ONYX-015 have also been demonstrated in mouse xenograft models using HLaC laryngeal carcinoma cells, RKO, HCT116, and SW620 cells (all colorectal carcinoma cells) [66, 73].

Interestingly, ONYX-015 works most effectively when injected directly into tumors and in combination with genotoxic agents, such as 5-fluoruracil (5-FU) or cisplatin [67]. These chemotherapeutic agents are commonly used to treat head and neck cancer patients. Unlike cisplatin or 5-FU alone, treatment with ONYX-015 alone increases survival times significantly [67]. However, the combination of cisplatin or 5-FU with ONYX-015 has been shown to be more effective than chemotherapy or virus treatment alone and results in a significant increase in the median survival in xenografted mice [67]. This synergistic effect could be achieved by intratumoral and intravenous administration of ONYX-015. The mechanism by which the combination of chemotherapy and ONYX-015 treatment improves efficacy is not well understood. Synergistic effects are most possibly due to chemosensitization and local bystander effects promoted through cytokines such as tumor necrosis factor [74] can be speculated.

Furthermore, it has been shown in vivo that ONYX-015 viral therapy can be combined with radiotherapy to improve tumor control beyond that of monotherapy [75]. Unlike other strategies that generate synergistic effects, the combined effects of ONYX-015 viral and radiation therapy appears to be additive in the tested tumor model.

Based on these studies and on the demonstration of safety in the cotton rat, which is partially competent for replication of human adenoviruses, ONYX-015 entered phase I clinical testing in April 1996.

Clinical studies on ONYX-015

Phase I trial for treatment of head and neck cancer

A total of 22 patients with recurrent head and neck cancer were enrolled into the clinical phase I study [76]. Eligibility requirements included histologically confirmed squamous cell carcinoma of the head and neck that was recurrent and refractory to radiotherapy and/or chemotherapy. The primary objective of this study was to determine the safety, feasibility, and efficacy of a single intratumoral injection of ONYX-015. Treatment was well tolerated, with the main toxicity being mild flulike symptoms. The maximum dose injected was 10^{11} plaque-forming units, and this did not cause any serious adverse effects. Viral replication was found in 4 of 22 patients treated, all of whom had p53 mutant tumors. Although using conventional response criteria no objective responses were observed, magnetic resonance imaging demonstrated tumor necrosis at the site of viral injection in five patients showing evidence of antitumor activity. All patients developed a rising neutralizing antibody response despite being immunosuppressed. Taken together, these results suggested that intratumoral administration of ONYX-015 is feasible, well tolerated, and associated with biological activity.

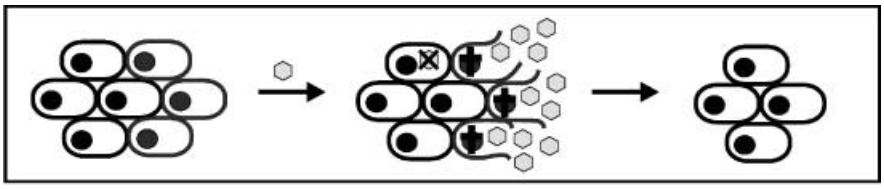
Phase II trials

ONYX-015 as monotherapy for recurrent head and neck cancer

To evaluate the safety, humoral immune response, replication, and activity of intratumoral injections of ONYX-15 in patients with recurrent squamous cell carcinoma of the head and neck, a clinical phase II trial was carried out [77, 78]. All patients enrolled had recurrence/relapse after conventional treatment and received ONYX-015 at a dose of 2×10^{11} particles (1×10^{10} pfu) via intratumoral injection for either 5 consecutive days or twice daily for 10 days during a 21-day cycle. Antitumor activity (as measured by >50% tumor destruction) was observed in approximately 14% of patients in both groups. Of the eight tumor regressions observed two partial and one minor regression were confirmed approximately 4 weeks later. A significant correlation was demonstrated between antitumoral activity (complete, partial, and minor responses) and presence of a p53 gene mutation. The therapy was well tolerated, with transient low-grade fever and injection pain as most frequent toxicities, which were all manageable on an outpatient basis. Neutralizing antibody titers either before or after treatment with ONYX-015 were not predictive for antitumor activity. However, despite the biological activity with ONYX-015 in this phase II trial clinical benefit was not seen in the majority of the patients [77, 78].

ONYX-015 in combination with chemotherapy for treatment of head and neck cancer

In vitro and mouse xenograft studies have shown synergistic efficacy of ONYX-015 in combination with chemotherapy, such as cisplatin or 5-FU, compared to treatment with ONYX-015 or chemotherapy alone [67]. A phase II clinical trial of intratumoral ONYX-015 injection in combination with intravenous cisplatin and 5-FU chemotherapy was performed in patients with recurrent squamous cell carcinoma of the head and neck [79]. Treatment caused tumors to shrink in 25 of the 30 cases evaluated. Objective responses (as measured by >50% tumor destruction) of injected tumors were observed in 63% of the evaluated patients with 27% (eight patients) complete responses. Based on an “intent-to-treat” analysis of all patients, the objective response rate of injected tumors was 53%. Six months after the end of the study none of the tumors with an objective response to the combined therapy had progressed, whereas all noninjected tumors treated with chemotherapy alone had pro-



virus	administration	cancer type	clinical phase I	II	III
ONYX 015	intratumoral injection + chemotherapy	head and neck			
	intratumoral injection + chemotherapy	pancreatic cancer			
	hepatic artery infusion + chemotherapy	liver metastases colorectal cancer			
	intratumoral injection + chemotherapy	sarcoma			
	intratumoral injection	malignant glioma			
	mouthwash	oral leukoplakia			

virus	administration	cancer type	clinical phase I	II	III
CV706	intratumoral injection	prostate cancer			
CV787	intratumoral injection	prostate cancer			
G207	intratumoral injection	glioblastoma multiforme			
Reovirus	intratumoral injection	advanced tumors			

Fig. 3 *Upper panel* Schematic principle of tumor-selective replication of ONYX-015. Both normal cells and tumor cells are infected by ONYX-015. While in normal cells no replication is supported, and infection is abortive (no production of new virus particles), cancer cells allow replication of ONYX-015. After lysis of the infected cancer cell, new infectious virus particles are released and spread to the neighboring tumor tissue. *Lower panels* Ongoing clinical trials using tumor-selective replicating viruses

gressed. The most common adverse events reported in this study were injection site pain and mucositis (often observed with 5-FU treatment), while flulike symptoms were less frequent than in the clinical studies using ONYX-015 as a single therapeutic agent [77, 78]. There was no correlation between response and baseline tumor size, baseline neutralizing antibody titer, p53 gene status, or prior treatment. Tumor biopsy specimens obtained after treatment showed tumor-selective viral replication and necrosis induction [79].

The reasons for a synergistic effect on the clinical response observed when combining ONYX-015 with standard chemotherapy are not well understood. Chemosensitization is a likely explanation in ONYX-015 treated patients. This is consistent with the observation that the patients suffering from multiple head and neck tumors [79], the majority of ONYX-015 injected tumors (9 of 11) regressed in combination with chemotherapy, whereas only a few noninjected lesions responded (3 of 11) [79]. Cellular and humoral immunosuppression is frequently observed with chemotherapy, and therefore improved virus replication may contribute to the synergistic effects.

Because of the promising results obtained from this clinical phase II trial, ONYX-015 entered phase III clinical testing for the treatment of recurrent head and neck cancer in December 2000. ONYX-015 combined with standard chemotherapy will be compared to chemotherapy alone to determine whether the combination therapy significantly improves durable tumor response and pro-

gression-free survival. The trial will take place at numerous places in Europe and the United States and will include approximately 300 patients.

Clinical trials on ONYX-015 for other cancers

Clinical phase I/II trials are currently being conducted evaluating ONYX-015 in patients with liver metastases of colorectal cancer and pancreatic cancer (Fig. 3). ONYX-015 is administered through hepatic artery infusion in patients with liver metastases of colorectal cancer. This allows simultaneous delivery of ONYX-015 to multiple tumors within the liver. ONYX-015 treatment is combined with administration of 5-FU and leucovorin. Patients with pancreatic cancer are treated with ONYX-015 via computed tomography guided intratumoral injections [80]. In two phase I clinical trials patients with sarcomas and glioblastoma are being treated with intratumoral injection of ONYX-015 (Fig. 3). Other indications include oral leukoplakia and cervical and bladder cancer. Also, intravenous infusion of ONYX-015 has been shown to be safe and feasible in patients with advanced metastatic carcinoma to the lung [81].

Other viruses used as oncolytic agents

Adenoviruses

Recently other tumor-selective adenovirus mutants have been reported in which essential viral genes were modified or deleted. These mutants proposed for specific replication are based on the deletion of the Rb-binding site of E1A [82, 83]. They are unable to induce resting cells to pass the G_2/M checkpoint and progress into mitosis. One of these mutants targeting the Rb pathway, AdΔ24, has been shown to produce antiglioma effects in vivo [83].

Furthermore, in several adenovirus vectors the transcription of essential viral genes is controlled by replacing the native viral promoters with tumor-specific promoters. Using this strategy a series of prostate cancer specific adenoviruses have been generated [84, 85, 86]. Promoters of prostate-specific genes, such as prostate-specific antigen (PSA) and human kallikrein 2, control the expression of the viral E1A and E1B proteins. The regulatory region of those prostate-specific genes contains several androgen-responsive elements and prostate-specific enhancers [86]. CN706, an adenovirus in which the *E1A* gene is driven by the PSA promoter, has been shown to destroy human PSA-positive cells 400 times more efficiently than PSA-negative cells and eliminates LNCaP (human PSA-positive prostate cancer cell line) xenografts in nu/nu mice with a single intratumoral injection [84]. CV764 contains both viral genes (*E1A* and *E1B*) under the control of prostate specific promoters. While the PSA promoter drives the gene *E1A*, the *E1B* gene is controlled by the promoter/enhancer of the human kallikrein 2 gene. CV764 kills PSA-positive cells 10,000 times better than PSA-negative cells but cannot eliminate distant preexistent LNCaP xenograft tumors in nu/nu mice by intravenous tail vein injection [85]. In both viruses, CN706 and CV764, the adenoviral E3 region, which is not essential for viral replication, has been deleted [84, 85]. However, recent studies demonstrate that the proteins encoded by the E3 region might play a role in assisting virus release and evading or slowing host immune responses to the virus [87, 88]. It was therefore of interest to construct an adenovirus which retains the entire E3 region, but replicates in a prostate specific manner: CV787 was generated using the rat probasin prostate-specific promoter, driving the E1A expression, and the prostate-specific enhancers controlling the *E1B* gene [85]. This virus contains the complete E3 region. CV787 replicates as does the wild-type adenovirus in cells that express PSA but is attenuated 10,000- to 100,000-fold with respect to replication in PSA negative cells. The addition of the adenoviral E3 region increased efficacy 10- to 100-fold both in vitro and in vivo. Most importantly, CV787 could eliminate preexistent, distantly located LNCaP tumors in nu/nu mice [85].

CV706 and CV787 are currently in phase I/II clinical trials for organ-confined and metastatic prostate cancer.

Using a similar strategy, adenoviruses have been constructed to express the *E1A* gene under the control of the α-fetoprotein promoter [89]. α-fetoprotein is expressed at high levels in hepatocellular carcinomas [90].

Herpes simplex virus

Another attractive virus for experimental tumor therapy is the genetically engineered herpes simplex virus (HSV) type 1 [91]. Since malignant gliomas are the most common primary malignant brain tumors and fatal despite aggressive therapies including surgery and radiotherapy, the search for alternative therapies is clearly needed [92]. HSV-1 replicates in neuronal tissue and has been shown to kill tumors derived from the nervous system. However, the wild-type HSV-1 can cause a fatal hemorrhagic encephalitis in humans [93]. Through deletion of both copies of the neurovirulence gene γ34.5 an avirulent version of HSV-1 can be generated [94, 95]. The second generation of HSV-1 vectors G207 comprises a multigene mutant with deletions not only at the γ34.5 loci but also a lacZ insertion in the ICP6 gene, allowing the tracking of viral replication [91]. The ICP6 gene encodes for the ribonucleotide reductase, which is essential for replication [96, 97]. Host gene expression in dividing cells is presumably able to complement the missing enzyme activity in *trans*, rendering the mutant virus G207 conditionally replicating in dividing cells. These multiple and large deletions/insertions make reversion to wild type highly unlikely. The ribonucleotide reductase mutation results in a hypersensitivity of G207 to antiviral compounds such as ganciclovir, which is already in clinical use [91]. Thus as an additional safeguard in the case

of encephalitis or excessive toxicity caused by the treatment with G207 an effective antiviral medication would be available. Furthermore, the mutant HSV-1 is characterized by a temperature sensitivity that would compromise viral replication in the presence of encephalitis and fever [91].

The safety of G207 inoculation has been demonstrated in a clinical phase I trial (1998–1999) which enrolled 21 patients suffering from malignant glioma. No patient developed HSV encephalitis, nor could any other toxicity be ascribed to treatment with G207 [98]. A phase II clinical trial using a combination of G207 and radiotherapy is currently in preparation.

"Armed" adeno- and herpesviruses

A natural extension of using engineered viruses is to combine these tumor-selective replicating agents with the delivery of therapeutic genes (Fig. 1) [99, 100]. This approach offers the potential advantage of delivering high concentrations of antitumor activity to cancer cells with minimal toxicity to normal tissue. These therapeutic genes encode for prodrug-converting enzymes (such as thymidine kinase), suicide genes, or immunostimulatory cytokines [such as interleukin (IL) 4, IL-12]. Prodrug-converting enzymes convert nontoxic prodrugs into cytotoxic metabolites, killing the prodrug enzyme- expressing cell as well as neighboring cells. Since many of these activated prodrugs target DNA replication, timing, and dosing of prodrug administration needs to be optimized to ensure efficacy of the treatment. It has been shown that addition of thymidine kinase to an E1B55K-deleted adenovirus mutant results in improvement in treating efficacy in combination with the prodrug ganciclovir in HT-29 colon cancer xenografted nu/nu mice [101]. Most importantly, the delivery was markedly better in replication-selective viruses than in replication-incompetent vectors. In another approach, an E1B55K-deleted adenovirus was developed containing a thymidine kinase fusion gene with cytosine deaminase, which was shown to be superior to each single agent alone [102]. Both "armed" adenoviruses showed enhanced antitumoral efficacy, and offer appealing options for improving efficacy.

Suicide genes have also been incorporated into HSV-1. In this HSV mutant the prodrug converting gene CYP2B1 replaces the HSV ribonucleotide reductase gene, rendering this mutant more effective against tumors when treated with the prodrug cyclophosphamide. The addition of cyclophosphamide had minimal effects on productive viral infection [103].

The IL-4 and IL-12 cytokine genes have also been introduced into the oncolytic HSV to couple tumor specific viral replication with a stimulation of the host immune response. IL-4 and IL-12 were chosen based on their ability to stimulate macrophage and T-cell proliferation and promote cytolytic activity of natural killer cells and cytotoxic T-cells, respectively. Including these immunostimulatory factors into HSV resulted in enhanced antitumor activity and prolonged animal survival compared with control parental viruses. In addition, an increased number of inflammatory cells in the tumor tissue was observed [99, 100]. Optimization of therapeutic gene expression levels and timing of prodrug administration are crucial to achieve maximal therapeutic benefit in this setting.

Reovirus

Human reovirus requires an activated Ras signaling pathway for infection and replication in cultured cells [104]. Restriction of reovirus is due to activation of the double-stranded RNA-activated protein kinase by early viral transcripts, which in turn inhibits the translation of these transcripts. An activated Ras pathway inhibits activation of RNA-activated protein kinase, thereby allowing viral protein synthesis and replication [104]. It has been shown that reovirus specifically targets tumors with a highly activated Ras signaling pathway. Although only about 30% of human cancers have activating mutations in the Ras gene itself, it is conceivable that more than one-half of all tumors have an activated Ras pathway due to activating mutations in genes up- or downstream of Ras [105, 106]. In xenograft models using human glioblastoma U87 cells tumor growth was substantially suppressed, and tumor regression was observed in four of five nu/nu mice after initial treatment. More importantly, it was demonstrated in immunocompetent mice using Ras-transformed syngeneic fibroblasts that the tumors regressed completely in six of nine reovirus-treated animals, despite of the host immune response [12].

Newcastle disease virus

NDV strain 73-T has been reported to be cytolytic to tumors cells [11]. Data from tissue culture and from xenograft models suggest that tumor cells are much more susceptible to NDV replication and virus-mediated cytolysis than normal cells [11, 13, 107, 108]. The selective sensitivity of tumor cells to NDV may be due to differential uptake, replication, or release of the virus. Since NDV is a potent inducer of tumor necrosis factor α, interferon, and IL-1, local production of these cytokines during NDV infection might contribute to the antitumor effect [11, 109]. The exact mechanism for tumor selective replication is unknown, but N-myc amplification seems to support it [107, 108].

Immune response to viral and tumor antigens

Several aspects of the immune response must be distinguished, in particular the immune response directed to viral and those directed to tumor antigens. Very little is known on this important subject in the context of replication-selective viruses, partly due to the limitations of preclinical models.

Some of our knowledge has been derived from clinical trials. In the head and neck cancer patients receiving intratumoral injections of ONYX-015 [76, 77, 78, 79], nearly all developed neutralizing antibodies. However, there is no correlation between titers of neutralizing antibodies and tumor response [76, 77, 78, 79]. It remains to be determined whether neutralizing antibodies will affect clinical efficacy in systemically delivered adenoviruses. Intravenous delivery of the HSV-1 mutant G207 in a mouse model resulted in elevated levels of complement and immunoglobulins in the serum capable of inactivating G207 [110]. Nevertheless, intravenously delivered G207 resulted in viral gene expression for 4 days in multiple organs and induced regression and cure of distant tumors [111]. Clearly, systemic delivery of virus remains a challenge for the future.

It has been shown that many tumors are potentially immunogenic, but antigen-presenting cells fail to recognize the antigen. An enhanced immune response to tumor antigens has been proposed after injection with several replication-selective adenoviruses [112], as the presence of the virus itself or cell debris of lysed cells would significantly improve antigen presentation, promoting antitumor immunity. Studies regarding this issue are in their early stages, but there is some evidence that the route of administration plays a role in determining the strength of the immune response [113].

Concluding remarks

Replication-selective viruses offer two appealing properties as agents for cancer therapy: they kill tumor cells selectively, and their replication leads to amplification of their oncolytic effects. Preclinical data suggest that efficacy may be further improved by tumor targeting strategies or “arming” replication-competent viruses with therapeutic genes. Clinical data of the first generation of replication-selective viruses are now rapidly accruing. A number of issues need to be addressed in more detail, such as role of the host immune response against viral and tumor antigens and the mechanism of synergy between virus therapy and chemotherapy. Also, viral receptor distribution on cancerous and normal tissues is likely to influence intratumoral virus spreading. The therapeutic index, and ultimately the clinical outcome, will depend on a complex balance between host and viral factors. The promising clinical results have fueled the hope that using replication-selective viruses may lead to an effective cancer therapy, and phase III clinical testing is underway.

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