

Oncolytic Adenoviruses for the Treatment of Human Cancer: Focus on Translational and Clinical Data

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Received June 30, 2010; Revised Manuscript Received December 2, 2010; Accepted December 2, 2010

Abstract: Although results of cancer treatment have improved steadily, metastatic solid tumors can be cured only rarely and therefore new modalities are needed. Tumors often become apoptosis-resistant and capable of excluding drugs during therapy. Similar mechanisms of resistance apply to many treatment regimens, and cross-resistance between different chemotherapeutics often limits the treatment options. Therefore, loss of efficacy may occur simultaneously for different chemotherapeutics. One experimental strategy with an increasing amount of clinical evidence is oncolytic viruses, which replicate preferentially in tumor cells by taking advantage of cancer-specific cellular changes. Adenoviruses are the most widely clinically used oncolytic agents. Replication of oncolytic virus *per se* kills tumor cells, but oncolysis can also activate the immune system, which may play a role in tumor control. Viruses can be modified in a variety of ways to improve their selectivity and efficacy. The adenovirus genome can be easily engineered to incorporate different tumor targeting mechanisms and therapeutic transgenes for improved antitumor properties. Here we review the available preclinical and clinical data on use of oncolytic adenoviruses in humans.

Keywords: Oncolytic adenovirus; cancer; gene therapy; virotherapy

Introduction

Eleven million new cancer cases and 7 million deaths were reported worldwide in 2002. Furthermore, nearly 25 million people were living with cancer. Aging and world population growth have led to a progressive increase in cancer burden: 15 million new cases and 10 million new deaths are expected in 2020.¹ Modern research tools have helped reveal new molecular mechanisms of carcinogenesis. Development of malignancies is known to be a multistep process involving

progressive changes in the genome. The discovery of mutations leading to activation of oncogenes and inactivation of tumor suppressor genes was a critical step in understanding the nature of carcinogenesis. Better understanding of the disease and emerging modern technologies have led to the development of more sensitive diagnostic methods and new treatment modalities. However, cancer is still usually incurable when diagnosed in the advanced metastatic stages.

One experimental strategy with an increasing amount of clinical evidence is the use of oncolytic adenoviruses.^{2,3} They take advantage of cancer-specific changes for preferential replication in tumor cells.^{4,5} The most critical requirements for an efficient vector for cancer treatment include efficient

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transduction and gene expression in target cells.⁶ An advantage of adenoviruses, as an oncolytic vector, is that they can infect cells regardless of cell cycling and induce an S phase like state so that viral replication can proceed.⁷ After the first round of replication, cancer cells are lysed and virus progeny are released to infect neighboring cancer cells. In principle, infection and replication can continue until the whole tumor mass is eradicated. Theoretically, virus may also reach distant metastasis by entering the bloodstream.⁸ Furthermore, oncolytic cell death *per se* seems to be an immunogenic phenomenon and can break tumor associated immunotolerance.^{9–12}

The first generation of oncolytic adenoviruses has completed clinical testing, and the safety has been found excellent. However, efficacy as a single agent remains modest at least when used in the context of advanced tumors. Several possible explanations have been suggested based on pre-clinical models. Intravenous administration of adenovirus to mice leads to induction of viral neutralizing antibodies, binding to blood cells, sequestration by macrophages and high liver tropism. All these decrease the amount of systemically available virus. Both micro- and macroenvironment of tumors further hinder viral spread. Currently, many approaches are aiming to solve these issues including studies addressing specificity, delivery, and potency of oncolytic adenoviruses. However, due to these issues, intratumoral and/or local injections are currently favored over

systemic delivery for clinical use. Demonstrating the potency of the approach, even first-generation viruses can be quite effective when combined with chemotherapy.^{3,13}

Adenoviruses as Gene Transfer Vehicles

Traditionally, the term gene therapy is used to indicate gene delivery for insertion of nucleic acids into cells of an individual to treat a disease. The therapeutic transgene can replace a defective gene (e.g., tumor suppressor gene for the treatment of cancer or delivery of functional gene into target tissue in monogenic diseases), or encode RNA or protein with a therapeutic function.

In the past, the majority of clinical applications have been based on replacement of nonfunctional genes in monogenic disorders, but currently most of the studies are focused on cancer. Viral vectors are by far the most popular vectors for gene delivery, adenoviruses being the most commonly used viruses (25% of clinical trials).¹⁴ Specifically, 75% of adenoviral gene therapy trials are for the treatment of cancer.

Several features of adenovirus make it a suitable vector for oncolytic virotherapy: the naturally lytic replication cycle, efficient production of stable particles, efficient gene transfer and capability for infecting both dividing and nondividing cells. Furthermore, adenovirus production to high titers is relatively easy. In terms of safety, the nonintegrating adenoviral genome stays episomal in the target cell, and thus insertional mutagenesis is unlikely. Subgroup C and serotype 5 adenoviruses (Ad5) are most often used in clinical applications, and their pathology is well understood. Wild-type Ad5 typically causes mild upper respiratory tract and eye infections, which usually resolve uneventfully in healthy individuals. Recombinant genomes within 105% of the wild type 36 kb genome are efficiently incorporated into virus capsids resulting in stable viruses. This allows insertion of therapeutic transgenes for enhanced oncolytic activity.

Transductional Targeting of Adenoviruses

Adenovirus entry into cells is a two-step process. Initial binding between fiber and primary receptor(s) on the cell surface is followed by secondary interactions between other capsid proteins and cell membrane components and leads to internalization of the virus. Ad5 primary receptor CAR (coxsackie- and adenovirus receptor) and integrin expression levels of the host cell are the main determinants of Ad5 infection capacity *in vitro*. Many types of malignant cells express low levels of CAR and are therefore resistant to

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adenovirus infection.^{15–20} Numerous applications aiming at increased tumor targeting by chemical, genetic, and adapter-based methods have been described earlier.^{21,22}

With regard to chemical infectivity enhancement, histone deacetylase inhibitor trichostatin A can upregulate CAR for enhanced infectivity of low CAR cells.^{23–25} When combined with oncolytic adenovirus dl520 (ONYX-015), trichostatin showed a significant effect on replication and cytotoxicity.²⁶

Transductional targeting aims to affect entry of vectors into cells and may utilize two distinct applications (Figure 1.) Targeting can add tropism to new targets and simultaneously detarget from natural receptors. Retargeting aden-

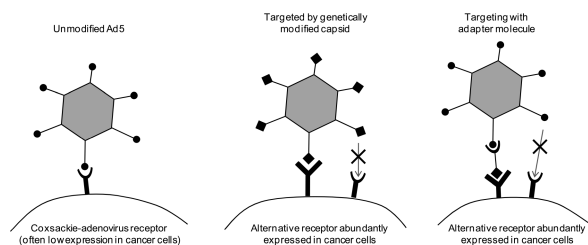


Figure 1. Transductional targeting of adenovirus to cancer cells via genetic modification of the virus capsid or by using adapter molecule.

ovirus can be useful for increasing the utility of the vector for cancer treatment. For example, inserting an RGD-containing peptide in the HI loop of the fiber knob allows the virus to enter cells through $\alpha_v\beta$ -integrins.^{27–29} $\alpha_v\beta$ -integrins are abundantly expressed on many cancer cells.^{30–32} Heparan sulfate proteoglycans (HSPGs) are common constituents of the extracellular matrix, have been found highly expressed in advanced tumors,^{33–38} and can be targeted using adenoviruses with a COOH-terminal polylysine tail.^{29,39,40}

Replacing part of the Ad5 capsid with non-serotype 5 components (= serotype fiber switching) can be utilized to circumvent CAR deficiency. Chimeric Ad5/3 virus has the serotype 5 capsid except for the fiber knob domain, which is from serotype 3 (Ad3). This modified knob region retargets

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virus to bind to Ad3 receptors,^{41,42} which are still unknown, but numerous studies suggest that they are abundantly expressed in many tumors.^{42–48} Also the serotype 35 fiber has been used to replace the Ad5 fiber for enhanced oncolysis. Chimeric Ad5 viruses containing Ad35 fibers

(Ad5/35) use CD46 as a receptor for infection of cells, which solves the problem of low CAR,⁴⁹ as many tumor types have been shown to express high levels of CD46.^{50–52} Components of serotype 35 may also protect chimeric vector from unspecific virus sequestration by blood components, including coagulation factor X.^{53,54} Furthermore, “directed evolution” has been used to create potent oncolytic adenoviruses against colorectal cancer. ColoAd1, a complex Ad3/Ad11p chimeric virus, was the initial oncolytic virus derived by this methodology and was found to be 100 times more selective for colon cancer cells in comparison to ONYX-015.⁵⁵

In addition to direct genetic modification of the vector, transductional targeting can be achieved by bridging the adenovirus vector and the cell surface receptor with an adapter-molecule such as bispecific antibodies,⁵⁶ cell-selective ligands such as folate⁵⁷ and chemical conjugates.⁵⁸ The two main goals of targeting are achieved by most of the

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currently studied adapter-based approaches: ablation of native CAR-dependent adenovirus tropism and formation of a novel tropism to specific cellular receptors. Chemically conjugated bispecific moieties consisting of an anti-knob Fab fragment and a natural ligand specific for cell surface receptor have the advantage that a variety of ligands, including vitamins, growth factors, antibodies, and peptides, can be chemically conjugated.²¹ A disadvantage of this approach is that the chemical conjugation results in a heterogeneous population of molecules. Furthermore, the yield of appropriately conjugated bispecific molecules can be relatively low.⁵⁹

To overcome the disadvantages of chemical conjugation, bispecific targeting moieties have been generated in the form of recombinant fusion proteins.⁵⁶ By these means, the expression and purification of a homogeneous population of retargeting molecules is possible. The principle of bispecific antibodies is that one site of the protein is directed against a viral capsid protein, while another site is specific for a cell surface molecule. A second variation of the approach utilizes a genetic fusion between the extracellular domain of CAR to a receptor-targeting moiety, yielding a truly targeted vector that blocks CAR binding. After adenovirus binding with CAR-ligand fusion protein, it will not be able to bind to its primary receptor. For example, a truncated, soluble form of CAR, sCAR, was fused to EGF. The bispecific fusion protein mediated EGFR-specific, CAR-independent adenovirus infection of target cells.⁶⁰

The ability of virus to reach organs through blood is reduced by liver sequestration, which can also contribute to toxic responses.^{61,62} Scavenger receptors on Kupffer cells, as well as natural antibodies and complement, mediate the

clearance of adenovirus.⁶³ Following adenovirus opsonization by plasma proteins, such as coagulation factors, liver uptake occurs in a CAR and integrin independent manner.⁶⁴ Binding between adenovirus fiber knob domain and coagulation factor IX (FIX) and complement component C4-binding protein (C4BP) provides a bridge for virus uptake through hepatocellular HSPGs and low-density lipoprotein (LDL)-receptor-related protein. Kupffer cell sequestration of adenovirus particles is likewise heavily dependent on viral association with FIX and C4BP.⁶⁵ Coagulation factor X (FX) has been shown to bind directly to the central depression of the hexon, but not to fiber. In comparison to other blood factors, FX shows the highest binding affinity to adenovirus and mediates efficient hepatocyte transduction.^{66,67} Transduction of hepatocytes is also facilitated by opsonization with other vitamin K-dependent coagulation factors,⁶⁸ and coagulation factor synthesis inhibition with warfarin reduces transduction of mouse liver, spleen and lung.⁶⁹

Polymers and Vehicles in Adenoviral Gene Delivery

Liver sequestration and induction of antiviral immune response might be partially avoidable by using carrier cells or coating agents for the delivery of Ad. Implantable delivery matrix, such as silica-based sol-gel polymer, has been successfully used to modify biodistribution and reduce drug toxicity for improved therapeutic efficacy of anticancer

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agents.⁷⁰ The goal of using a delivery matrix is to reduce toxicity and off-target delivery by allowing higher local drug concentration, longer target exposure and spontaneous slow degradation of the implant. Implantable silica matrix has been used in the context of oncolytic adenoviruses and resulted in prolonged survival of intraperitoneal orthotopic tumor-bearing mice. In addition, delivering the virus in silica had a favorable effect on anti-adenovirus immune response.⁷¹ A further example of a polymer for disguising the virus from the immune system is polyethylene glycol,⁷² which has been shown to reduce blood clearance rate but also infectivity.⁶²

Another approach for increased tumor transduction utilizes mesenchymal stem cells (MSCs). It has been previously suggested that MSC have tumor tropism.⁷³ Intravenous administration of MSCs preloaded with oncolytic adenovirus allowed release of the virus into advanced orthotopic breast and lung tumors and increased survival of mice. In contrast, prevalent liver transduction was demonstrated if the same dose of virus was injected without MSCs.⁷⁴ This approach has been tested also in the context of humans, with promising preliminary results in delivery of oncolytic adenovirus in chemotherapy refractory neuroblastoma patients.⁷⁵ The use of neural stem cells has been proposed to target intracranial glioma, and improved intratumoral distribution was seen in comparison to virus alone injection.⁷⁶ In another approach, cancer cells have been suggested a useful tumor targeting

vehicle and homing to metastasis was demonstrated after intravenous injection.⁷⁷

Transcriptional Targeting of Adenoviruses

Transcriptional targeting does not change the tropism of viruses but restricts gene expression to target cells. One possibility to target cancer gene therapy to tissues and to limit off-target toxicity is the use of tissue-specific promoters (TSPs). This approach has been used for mutation compensation or delivery of prodrug-converting enzymes.⁷⁸ Another application of TSPs is the use of tumor-specific promoters in the context of oncolytic viruses. When critical regulators of viral replication are controlled by TSPs, replication is restricted to tissues where this particular promoter is active. E1A, as the main regulator of adenovirus replication, offers the natural choice to control virus replication via TSPs but also other adenovirus genes, such as E1B, E2 and E4 can be placed under control of TSPs.⁷⁹

Numerous TSPs based on e.g. prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), and melanoma differentiation marker tyrosinase enhancer/promoter have been tested successfully, and tumor-specific replication of these constructs has been demonstrated in animal models.^{80,81} As an example, hepatocellular carcinoma targeted AFP promoter driven oncolytic adenovirus CV890 was able to eliminate xenografts in mice, when administered in combination with doxorubicin.⁸² Further examples of suggested cancer-specific promoters are tyrosinase, human telomerase (hTERT),^{83,84} and cyclooxygenase-2 (Cox-2) promoters.^{85–87} Furthermore, adenoviral replication can be tightly directed by controlling two genes

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simultaneously with a single enhancer or enhancer/promoter, or with promoters controlled by the same transcription factors.⁸⁸

Conditionally Replicating Adenoviruses (CRAds)

Increased understanding of adenovirus replication and its interactions with cellular proteins have inspired the construction of conditionally replicating adenoviruses (CRAds). Infection of tumor cells results in replication, oncolysis, and subsequent release of the virus progeny. Normal tissue is spared due to lack of replication. These type 1 CRAds feature loss-of-function mutations in the virus genome, which are compensated by cellular factors present in cancer cells but not in normal cells.

“D24”-type adenoviruses, such as Ad5-D24, have a 24 bp deletion in pRb binding site in the constant region 2 of E1A. The tumor specificity of D24 is based on the inability of the modified E1A protein to bind cellular Rb protein. In normal cells, this binding is required for effective virus replication. Binding of Rb by E1A releases E2F, which activates the E2 viral promoter and initiates replication. However, in tumor cells, where the Rb-p16 pathway is inactive, abundant E2F is available and E1A-Rb binding is not necessary.^{89,90} It has been suggested that most human

cancers are deficient in this crucial pathway^{91,92} that regulates the G1-S checkpoint.

Oncolytic adenovirus dl1520 (ONYX-015) contains two mutations in the gene coding for the E1B-55kDa protein. The functional form of this protein binds to and inactivates cellular tumor suppressor p53 in virus infected cells for induction of S-phase, which is required for effective virus replication. Mutated E1B-55kDa in ONYX-015 is unable to bind p53, and therefore the virus should only replicate in cells lacking functional p53, thus including most human tumors.⁹³ Of note, even in tumor cells, replication of ONYX-015 is severely impaired compared to wild type virus, which may be due to defective late virus mRNA transport caused by absence of E1B-55kDa.⁹⁴ p53-selectivity of the virus has also been disputed,⁹⁵ and a recent study suggested that late mRNA transport might be more important than p53 status.⁹⁶ It was subsequently reported that viral 100K expression may occur differently in permissive versus nonpermissive cell lines in the absence of E1B-55kDa.⁹⁷ Furthermore, heat shock proteins and late viral RNAs share similar 5'UTRs, and therefore heat shock has been shown to rescue late viral RNA export and improve ONYX-015 replication in refractory cells.⁹⁷ Interestingly, this was hypothesized to explain why patients with virus induced fever getting no antipyretic drugs showed better response in H101 clinical trial (virus similar to ONYX-015).³ One controversy involves replication in some cultured cells lacking p53 mutations. Infection with E1B-55K mutated viruses leads to induction, but not activation, of p53 in primary cells, and therefore productive replication might occur.⁹⁸

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Adenovirus containing deletions in virus-associated I (VAI) and VAI RNA genes has shown selective replication in Ras mutation positive tumor cells.⁹⁹ The mRNA translation promoted by VAI and VAI RNA genes can be phenocopied in tumor cells with the activation of the Ras pathway.⁹⁹ Ras mutations are common in different types of tumors^{100–102} and are associated with aggressive phenotype and poor prognosis.¹⁰³

Type 2 CRAds contain tumor-specific promoters to replace endogenous viral promoters. These viruses are discussed further in the section Transcriptional Targeting of Adenoviruses.

Armed Oncolytic Adenoviruses

The potency of oncolytic adenovirus can be increased by arming the virus with a therapeutic transgene. Several approaches have been tested in preclinical models as well as in clinical trials. Tumors are heterogeneous and compartmentalized containing various obstacles, including hypoxic areas and regions of increased hydrostatic pressure, which hinder efficient spreading of virus. Therefore replication-mediated oncolysis alone may not be able to eradicate advanced tumor masses. To overcome such obstacles, targeting not only tumor cells but also normal cells within the tumor is of interest. Tumor neovasculature is one of the noncancer cell targets in cancer gene therapy. Antiangiogenic approaches include soluble vascular endothelial growth factor (VEGF) receptors and antibodies that can be expressed by adenovirus. Intravenous or intratumoral injection of soluble VEGF receptor producing vectors have been reported to result in pronounced tumor growth inhibition and prolonged survival in mice.^{104–107}

Degradation of tumor stroma can be facilitated by proteases coded from viral transgenes. In addition to treatment benefit from disruption of tumor structure by the protease, oncolytic activity is enhanced due to increased viral spread-

ing. Furthermore, no significant toxicity has been associated in this application.^{108,109} In another approach, coadministration of matrix-modifying metalloproteinases or bacterial collagenase together with oncolytic vectors has been shown to improve distribution and efficacy of virus.^{110,111}

An extensively studied approach called suicide gene therapy involves the tumor-targeted delivery of genes encoding enzymes that convert intravenously delivered prodrugs into toxic metabolites. By these means, a high concentration of toxic drug is achieved locally at the tumor site while a low systemic level of the active compound reduces toxic effects often seen with conventional chemotherapy. Studies utilizing oncolytic viruses coding for prodrug-converting enzymes have shown mixed results as toxic metabolites may hinder viral replication.¹¹² Nevertheless, combination therapy approaches with oncolytic adenoviruses have sometimes shown enhanced antitumor efficacy *in vivo* compared to the virus alone.¹¹³ The combination of cytosine deaminase (CD) and HSV-1 thymidine kinase (TK) gene delivery is an

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example of double suicide gene therapy for improved therapeutic outcome.¹¹⁴ Radiation therapy has been successfully combined with double suicide gene therapy mediated by oncolytic adenovirus in an orthotopic mouse prostate cancer model.^{114,115} D24-Type oncolytic adenovirus has been also combined with a suicide gene therapy by utilizing prodrug converting enzyme carboxylesterase/irinotecan (CE/CPT-11) system. CE converts CPT-11 into much more potent SN-38, which in this case augments the cytotoxicity of the virus for colon cancer cells.¹¹⁶ However, in the context of highly potent oncolysis, a suicide gene system such as TK/GCV may not add antitumor activity.¹¹²

Immune stimulatory cytokines or chemokines can be locally expressed in tumors with oncolytic viruses.^{117–120} For example, Ad5-D24-GMCSF expresses granulocyte-macrophage colony stimulating factor (GMCSF), a potent immune activator with the ability to mediate antitumor effects by recruiting natural killer cells and by priming tumor-specific CD8+ cytotoxic T-lymphocytes through antigen

presenting cells (APCs). Furthermore, oncolytic tumor cell killing can produce a potent costimulatory danger signal and lead to release of tumor epitopes for APC sampling. Ad5-D24-GMCSF was reported to induce both tumor- and virus-specific immunity in patients. Intriguingly, responses were seen in both injected and noninjected tumors.¹²¹ Similar approaches with oncolytic herpes (clinicaltrials.gov NCT-00769704) and vaccinia viruses¹²² coding for GMCSF have already progressed to phase 3 studies.

Immune Response

Even if clinical trials have shown good safety for oncolytic adenoviruses, limitations for efficacy have been recognized. Both innate and adaptive immune systems efficiently recognize adenovirus as a pathogen,¹²³ which may lead to elimination of virus and reduced antitumor efficacy. Virus interaction with leukocytes and endothelial and epithelial cells triggers immune response against virus. Tissue macrophages are derived from monocytes in the bloodstream. After entering tissues, they differentiate into mature phagocytic cell populations capable of clearing systemically injected virus from the bloodstream. These cells, along with activated dendritic cells (DCs) in the spleen, have a crucial role in provoking virus-induced inflammatory response.¹²⁴

Upon natural adenovirus infection, clearance of the virus results from combined innate and adaptive immune response.¹²⁵ It is not clear if high immunogenicity of adenovirus is an advantage or disadvantage in terms of treatment efficacy. The efficacy of the vector might be reduced due to induction of immunity, but on the other hand, neutralizing antibodies may increase safety by preventing vector from spreading into normal organs.^{126,127} Fortunately, virus-induced immune response can be utilized to synergize with the antitumor activity of the virus. Many applications of

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cancer immunotherapy rely on this approach. The inflammatory response is useful for antigen presentation and helps to reveal the hidden tumor antigens to antigen presenting cells for activation of tumor-antigen-specific T cells.¹²⁸ Oncolysis also creates a strong danger signal required for breaking tumor-induced tolerance and subsequent induction of productive systemic antitumor immunity.¹²⁹

Thus, the goal is to induce systemic tumor-specific immunity. In contrast, acute immune responses are the main safety concern regarding adenoviral gene therapy. This was tragically experienced in a phase I study where one patient with ornithine transcarbamylase deficiency died as a result of treatment. The vector used in this study was based on adenovirus serotype 5 and was deleted in E1 and E4 genes. A massive cytokine response and disseminated intravascular coagulation were reported following adenovirus administration.¹³⁰ However, it is noteworthy that 16 000 patients treated with adenoviral gene therapy have proven adenovirus to have a good safety profile compared to most conventional therapies and in fact no mortality has been reported in the context of cancer therapy, which is in striking contrast with the 1–5% mortality associated with chemotherapy or surgical oncology.¹³¹

The major adenovirus capsid protein, hexon, is the most important component in both humoral and cellular adaptive immune responses.^{132–137} Long-term expression of transgene is often attenuated due to humoral and cellular immune

responses, as described in different animal models.^{138–142} Especially systemic readministration of the vector may be compromised due to induction of anti-adenoviral neutralizing antibodies (NABs).¹⁴¹ “Gutless” vectors lacking all viral genes induce weaker adaptive response, but responses to capsid proteins are not attenuated.¹⁴³ The adaptive response requires the integration of both adenovirus-specific memory CD4+ and cytotoxic CD8+ T cell (CTL) responses. Conserved epitopes within the adenovirus capsid (mostly hexon) are the main targets for adenovirus-specific CTLs. CTLs attempt to kill infected cells and disrupt the adenovirus life cycle before progeny viruses are assembled by using multiple mechanisms including perforin, Fas-L and TNF α .¹⁴⁴

Oncolytic viruses can be combined with immune modulatory agents for enhanced viral spread, transgene expression and antitumoral efficacy. For example, cyclophosphamide has been used in combination with oncolytic adenovirus in animal and human studies to suppress regulatory T cells (Treg) and increase antitumor immune reactions.^{11,121,145–147}

In terms of clinical use of oncolytic adenoviruses, heavily pretreated cancer patients might be partially immune sup-

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pressed, which could theoretically increase the risk for virus replication associated side effects. On the other hand, attenuation of the antiviral response could prolong oncolytic replication and therefore improve potency.¹⁴⁸ To address this, we performed a retrospective case control study to assess the safety of corticosteroids, potentially immune suppressive agents, in cancer patients receiving oncolytic virotherapy, with or without concurrent low-dose cyclophosphamide. We found that coadministration of glucocorticoids and oncolytic adenovirus was safe in cancer patients.¹⁴⁹

Syrian hamsters are semipermissive for human adenovirus replication and therefore comprise a valuable immune competent model for evaluating adenovirus induced immune responses. It has been shown that pre-existing immunity to Ad5 does not reduce antitumor efficacy of intratumoral injection of serotype 5 adenovirus in hamsters, but immunity reduces vector spillover from the tumor to nontarget organs.¹²⁶ Furthermore, Syrian hamsters have been used to evaluate the efficacy of anti-adenoviral drugs for development of antiviral treatments,¹⁵⁰ and chlorpromazine and cidofovir have been shown to efficiently limit virus replication in this immune competent model.¹⁵¹

Immunological Obstacles to Systemic Administration

No strong evidence of efficacy from systemically administered oncolytic adenovirus exists, although some encourag-

ing preliminary results have been obtained in a small number of patients treated with advanced agents.^{8,146} In contrast, efficacy has been modest for attenuated first-generation adenoviruses.^{10,152} It is likely that a number of different mechanisms contribute to neutralization of systemically administered virus, including classic opsonization and aggregation of virus at the cell surface (which may impede proper recognition). Some inhibitory actions may even take place after virus-antibody complex entering the host cell.¹⁵³ Furthermore, complement and binding to blood cells can play a role in thwarting infection.¹⁵⁴

As the majority of adults have been exposed to the most widely used serotype 5 adenovirus (Ad5), the immune system is primed to rapidly produce NABs on re-exposure. An ability of NAB to directly block adenovirus vector in readministration has been demonstrated with passive transfer of serum from treated to naive animals.^{28,141} If virus replication is attenuated due to genetic engineering, the balance between immune response and oncolysis can favor the former. No data suggests that a high titer of NAB would compromise local injection, but it may limit systemic delivery. To overcome this, transient removal of pre-existing antibodies at the time of virus administration has been suggested.¹⁵⁵

Changing the adenovirus fiber knob for readministration allowed escape from NABs in immune competent mice.²⁸ The same approach was subsequently translated into humans with a similar outcome, suggesting that seroswitching might be interesting to study in repeated dosing regimens.¹⁵⁶ In line with this, an earlier study showed that humoral anti-adenoviral immunity could be avoided in a repeated dosing regimen by changing the entire serotype.¹⁵⁷ Other suggested ways of avoiding pre-existing antibodies are induction of

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immunological tolerance,¹⁵⁸ and the use of polyethylene glycol^{72,159} or silica gels⁷¹ to mask vector. Transient immunosuppression during initial administration of adenovirus might perhaps prevent a rise in antibody titer, but it would not be expected to suppress the levels of pre-existing antibodies.¹⁵⁵ Overall, it is not clear if pre-existing immunity impairs or helps the efficacy of an oncolytic virus. Also, much remains to be studied vis-à-vis interaction of adenovirus and blood cells. For example, virus may be able to utilize binding to red blood cells, platelets and leukocytes to avoid antibodies and to “hitchhike” to target organs. Also, it is not known if the tremendous amount of virus shed into blood for months after intratumoral injection is infectious or not.^{121,146,160–163}

Interestingly, a recent report showed that the oncolytic effect of modified HSV was enhanced in HSV-1 seropositive mice, possibly due to interferon (IFN)- γ mediated tumor cell killing.¹⁶⁴ Furthermore, preimmunization of mice has been shown to increase the antitumor potency of intratumorally injected adenovirus in the presense of NAbs.¹²⁹

Following intravenous administration of adenovirus to mice, adenovirus is rapidly cleared from the circulation¹⁶⁵ due to uptake by hepatic macrophages (Kupffer cells)¹⁶⁶ and

by transduction of hepatocytes.¹⁶⁷ Circulating platelets rapidly bind Ad5 after systemic administration, which leads to their activation/aggregation and subsequent entrapment in liver sinusoids. Kupffer cells take up virus–platelet aggregates for degradation. On the other hand, depletion of platelets prior to treatment has been shown to reduce adenovirus sequestration in organs.¹⁶⁸ GdCl₃ can be used to deplete Kupffer cells, and increased viremia following this treatment has been demonstrated *in vivo*.⁶² Preinjecting polyinosinic acid poly(I), a ligand for scavenger receptor, has been used to reduce the Kupffer cell uptake and increase the half-life of circulating adenovirus *in vivo*.^{40,69,169} Taken together, preventing anti-adenovirus NAbs or detargeting virus particles from liver may make the virus more applicable for systemic use. However, some evidence suggests also that NAbs might be beneficial for antitumor efficacy in local treatment.^{121,146,160–162} Furthermore, the relevance of liver uptake has only been studied in mice.

Cancer Stem Cells and Oncolytic Adenoviruses

The initiation and growth of tumors has been suggested to be controlled by specific subpopulation of malignant cells within tumors, so-called cancer-initiating cells or cancer stem cells (CSC).^{170–177} Putative CSCs share some features with

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their normal counterparts^{178,179} and have been shown to be remarkably resistant to radiation and chemotherapy.¹⁸⁰ Due to this treatment resistance, CSCs remaining after apparently curative treatment may eventually result in relapse. Furthermore, CSCs from a primary tumor may emigrate to distal sites and create metastases.¹⁸¹

Even though CSCs have multiple defense mechanisms, such as efflux pumps and defective apoptotic signaling¹⁸² to make them resistant to conventional cancer therapies, the entry of adenovirus into CSC by infection has not been reported restricted. Oncolytic adenoviruses can be engineered to infect CSCs by utilizing lineage-specific cell surface markers, dysfunctional stem cell-signaling pathways, or upregulated oncogenic genes.¹⁷⁷ Indeed, oncolytic viruses are the first approach shown to be effective against tumor-initiating cells.^{39,85} However, the infectivity of CSCs with Ad5 based vectors might be compromised due to low or missing expression of CAR in the surface of CSCs, as has been suggested for normal mesenchymal stem cells.⁷⁴ Fortunately, several studies have shown that the overall problem of CAR deficiency in stemlike cells can be overcome by using capsid modified adenoviral vectors.^{39,74,183,184} In addition, single chain monoclonal antibodies or other

bispecific adapter molecules could be used to transductionally target adenovirus to specific cell surface proteins.¹⁸⁵

Clinical Use of Oncolytic Adenoviruses

Clinical examples of oncolytic adenovirus use are listed in Tables 1 and 2. A phase I study with E1B-55kDa gene-deleted ONYX-015¹⁵² established clinical proof-of-concept for oncolytic virotherapy. In this study virus was injected directly into head and neck tumors.¹⁸⁶ Treatment with ONYX-015 was well tolerated and showed localized efficacy in head and neck cancer patients as a single agent.¹⁸⁷ Initially, patients with advanced incurable cancers were enrolled, but treatment of patients with premalignant conditions was started after demonstration of safety.¹⁸⁸ Overall, ONYX-015 proved to be safe, and even if it showed some activity when injected intratumorally, it was inefficient as a single agent.¹⁸⁹

To improve antitumor efficacy, ONYX-015 was the first oncolytic virus combined with chemotherapy in clinical trial. Oncolytic viruses are known to be synergistic with many chemotherapeutics and radiation therapy.^{72,190} Local responses were achieved with direct injection of the virus when combined with systemic cisplatin and 5-fluorouracil (5-FU)

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Table 1. Unarmed Oncolytic Adenoviruses in Clinical Studies

virus/treatment agents	genetic modification	phase	route of administration	cancer type	efficacy/no. of patients	ref
ONYX-015	E1B-55kDa deletion	I	i.t.	SCCHN	2/22	186
ONYX-015	E1B-55kDa deletion	I	i.t.	pancreatic cancer	0/23	201
ONYX-015	E1B-55kDa deletion	I	iv	cancer metastatic to the lung	0/10	202
ONYX-015	E1B-55kDa deletion	I	ip	ovarian cancer	0/16	203
ONYX-015	E1B-55kDa deletion	I	iv + i.t.	HCC	1/5	204
ONYX-015	E1B-55kDa deletion	I	i.t.	glioma	3/24	205
ONYX-015 + etanercept	E1B-55kDa deletion	I	iv	advanced cancers	0/9	206
CV706	PSA promoter controlling E1A	I	i.t.	prostate cancer	5/20	191
ONYX-015 + 5-FU	E1B-55kDa deletion	I–II	i.t. + iha + iv	HCC/colorectal cancer metastatic to liver	3/16	207
ONYX-015 + 5-FU + leukovorin	E1B-55kDa deletion	I–II	iha	metastatic colorectal cancer	2/24	208
ONYX-015	E1B-55kDa deletion	II	i.t.	SCCHN	5/40	209
ONYX-015 + cisplatin + 5-FU	E1B-55kDa deletion	II	i.t.	SCCHN	19/37	190
ONYX-015 + gemcitabine	E1B-55kDa deletion	I–II	i.t.	pancreatic cancer	2/21	210
ONYX-015	E1B-55kDa deletion	II	iv	metastatic colorectal cancer	0/18	211
H101 + cisplatin/ adriamycin + 5-FU	E1B-55kDa deletion	III	i.t.	SCCHN	71/160	212
ONYX-015 + MAP chemotherapy	E1B-55kDa deletion	I–II	i.t.	sarcoma	1/6	213
CG7870	tumor-specific	I	iv	hormone refractory	5/23 decline in PSA, 3/8 at highest dose levels	8
(CV787)	promoters driving E1A and E1B			prostate cancer		
Ad5/3-Cox2L-D24	5/3 chimeric capsid, 24 bp deletion in E1A, Cox2L promoter driving E1A	ATAP	personalized i.t. + iv	advanced solid tumors	10/14	162
ICOVIR-7 + most patients received low-dose cyclophosphamide to downregulate T-Reg	RGD-capsid modification, 24 bp deletion in E1A, Cox2L promoter driving E1A	ATAP	personalized i.t. + iv	advanced solid tumors	9/17	146
Ad5-D24-RGD	RGD-capsid modification, 24 bp deletion in E1A	ATAP	personalized i.t. + iv	advanced solid tumors	3/8	161

^a Abbreviations: 5-FU, 5-fluorouracil; ADP, adenoviral death protein; ATAP, Advanced Therapy Access Program (www.oncos.com); CD, cytosine deaminase; GCV, ganciclovir; HCC, hepatocellular carcinoma; MAP, mitomycin-C–doxorubicin–cisplatin; iha, intrahepatic artery; i.t., intratumoral; iv, intravenous; mitomycin-C–doxorubicin–cisplatin; pfu, plaque forming unit; PSA, prostate-specific antigen; SCCHN, squamous cell cancer of the head and neck; TK, tyrosine kinase; T-Reg, regulatory T-cell; vp, viral particle.

Table 2. Armed Oncolytic Adenoviruses Used in Cancer Patients

transgene	function of the transgene	genetic modifications	virus	phase	concomitant treatments	cancer type/route of administration	efficacy/number of evaluable patients	ref
bacterial CD/HSV-1 TK	prodrug conversion: CD, 5-FC to 5-FU; TK, GCV to toxic metabolite	CD/HSV TK fusion gene in E1; E1B-55kDa deletion	Ad5-CD/TKrep	I	5-FC + GCV + radiation therapy	prostate cancer/intraprostatic	15/15 (PSA ↓)	72
yeast CD/HSV-1 TK	prodrug conversion: CD, 5-FC to 5-FU; TK, GCV to toxic metabolite	CD/HSV TK fusion gene in E1; ADP in E3	Ad5-yCD/mut TK _{SR39} rep-ADP	I	5-FC + GCV + radiation therapy	prostate cancer/intraprostatic	9/9 (PSA ↓)	214
+ GCV/5-FC + radiation	prodrug conversion: CD, 5-FC to 5-FU; TK, GCV to toxic metabolite	CD/HSV TK fusion gene in E1; ADP in E3	Ad5-yCD/mut TK _{SR39} rep-ADP	II–III	5-FC + GCV + radiation therapy	prostate cancer/intraprostatic	not available yet	<i>b</i>
human GM-CSF	immune stimulation	24 bp deletion in E1A; GM-CSF in E3	Ad5-D24-GMCSF	ATAP	none	advanced solid tumors/i.t. + iv	8/16 (CR/MR/SD)	193
human GM-CSF	immune stimulation	5/3 chimeric capsid; 24 bp deletion in E1A; GM-CSF in E3	Ad5/3-D24-GMCSF	ATAP	low dose cyclophosphamide	advanced solid tumors/i.t. + iv	8/12 (MR/SD)	194
human GM-CSF	immune stimulation	RGD-capsid modification; 24 bp deletion in E1A; GM-CSF in E3	Ad5-RGD-D24-GMCSF	ATAP	low dose cyclophosphamide	advanced solid tumors/i.t. + iv	3/6 (SD)	161
human GM-CSF	immune stimulation	hTERT promoter in E1A; GM-CSF in E3	KH901	I	none	head and neck cancer/i.t.	12/19 (SD)	120
HSP70	immune stimulation	HSP70 in E1; E1B-55kDa deletion	H103	I	none	advanced solid tumors/i.t.	13/27 (PR/MR/SD)	215

^a Abbreviations: CD, cytosine deaminase; HSV-1, herpes simplex virus-1; TK, thymidine kinase; 5-FC, 5-fluorocytosine; GCV, ganciclovir; ADP, adenoviral death protein. ^b clinicaltrials.gov NCT00583492; GM-CSF, granulocyte macrophage colony-stimulating factor; HSP70, heat shock protein 70; ATAP, Advanced Therapy Access Program (www.oncos.com); hTERT, human telomerase reverse transcriptase; i.t., intratumoral; iv, intravenous; CR, complete response; PR, partial response; SD, stable disease; MR, minor response.

chemotherapy.¹⁹⁰ Unfortunately, the US phase III clinical trial of head and neck carcinoma in combination with chemotherapy was halted in 2003 due to unresolved funding problems.

By 2005, a similar virus (H101) was constructed in China and rapidly taken through phase 1–3 testing and eventually approved for treating advanced head and neck carcinoma in combination with chemotherapy (5-FU + cisplatin). H101 became the first oncolytic virus product approved by a governmental agency for human use.³ Results similar to what has been reported earlier in the phase II trial combining ONYX-015 and chemotherapy were achieved with H101 in combination with chemotherapeutics.¹⁹⁰

Radiation therapy was first combined with an oncolytic virus in a study using replicating adenovirus, Ad5-CD/TK, which has an E1B deletion and carries a fusion gene expressing two prodrug-activating enzymes, CD from *Escherichia coli* and HSV-TK. Combination therapy with a virus, prodrugs (5-FC and valganciclovir), and radiation was safe and effective, although the relative merits of the different modalities are difficult to distinguish in a nonrandomized setting.⁷² Therefore, it is important that the same investigators have subsequently started a randomized phase 3 study (clinicaltrials.gov NCT00583492) to evaluate the utility of adding oncolytic adenovirus to radiation and hormonal therapy in the context of first line therapy of high risk prostate cancer.

Viruses carrying cancer-specific promoters to drive E1A have also been well tolerated in patients.^{162,191} Furthermore, transcriptionally targeted oncolytic adenoviruses expressing immunostimulatory cytokine GM-CSF showed good safety and some early evidence of efficacy in preclinical¹⁹² and phase I studies.¹²⁰ Safety and preliminary efficacy have been also reported following treatments with to a D24-type transcriptionally targeted virus carrying GM-CSF gene, and a similar virus with 5/3 serotype chimeric capsid.^{193,194} Further clinical evidence regarding other capsid modified, transductionally targeted oncolytic adenoviruses is currently emerging. Treatments of refractory solid tumors with armed and unarmed integrin-targeted viruses have been reported to result in good safety, and there is some evidence of activity.^{146,161}

Future Perspective

Testing of oncolytic adenoviruses in clinical trials is performed mostly in patients with advance refractory disease where no curative conventional treatment options are available. Like other modalities, oncolytic viruses might show better efficacy if earlier stage patients with less refractory disease were treated. Accordingly, recent studies have shown that an important part of the antitumor activity of these agents

is associated with immunological responses, and advanced high mass tumors are typically quite immune suppressive.¹⁹⁵ Also the use of oncolytic viruses as an adjuvant therapy after surgical removal of the tumor seems an attractive approach. At the end of the day, a minimal tumor load situation might be optimal for maximum benefit.

Best results with oncolytic viruses are probably ultimately achieved in combination therapy regimens because of lack of overlapping side effects and synergy with many chemotherapeutics or radiation,^{196–198} and many promising new approaches are currently under investigation. For example, serial treatments with different oncolytic viruses, with different transductional profiles, could be beneficial to optimize the relative advantages of each virus while partially avoiding potentially problematic NAb.¹⁵⁶ Some of the most promising approaches for enhanced antitumor efficacy of oncolytic virotherapy are related to redirection and augmentation of immunological antitumor responses. In fact, it has been proposed that the presence of tumor-infiltrating lym-

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phocytes is a strong prognostic factor associated with both freedom from disease and overall survival for tumors in general.¹⁹⁹

As for all cancer treatments, responses to oncolytic virus therapy vary from one individual to another and the optimal outcome might be achieved by developing individually personalized oncolytic virus medicine. For instance, pre-treatment tumor biopsies could be used to study the effects of different viruses *ex vivo* to find the most suitable virus for tumor eradication.^{193,194} A huge effort has been recently made for the identification of molecular markers to be used in optimized and tailored treatment regimens.²⁰⁰ Comparison between treatment efficacy and RNA profiles from the tumors before and after virus treatment might provide valuable information about who is likely to respond and to what kind of treatment, and would allow adjustment of the treatments accordingly.

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Acknowledgment. A.H. is K. Albin Johansson Research Professor of the Foundation for the Finnish Cancer Institute. A.H. is founder and shareholder in Oncos Therapeutics Inc. This paper was supported by the European Research Council, EU FP6 APOTHERAPY and THERAD-POX, ASCO Foundation, HUCH Research Funds (EVO), Sigrid Juselius Foundation, Academy of Finland, Biocentrum Helsinki, University of Helsinki.

MP100219N

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