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Current developments in adenovirus-based cancer gene therapy

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Abstract

Adenovirus (Ad)-based cancer gene therapy is a promising, novel approach for treating cancer resistant to established treatment modalities. Unfortunately, the efficacy of nonreplicative first generation Ads was low and data from clinical trials were disappointing. To address this problem, conditionally replicating Ads have been constructed. Infection of tumor cells with conditionally replicating Ads results in tumor-specific replication, subsequent oncolysis and release of the virus progeny. Recently, it has been suggested that the low expression of the coxsackie–Ad receptor is the rate-limiting factor for infectivity with serotype 5 (Ad5). Unfortunately, coxsackie–Ad receptor expression is highly variable and often low on many tumor types. Consequently, molecular strategies have been applied for the development of coxsackie–Ad receptor-independent oncolytic Ads. This review describes recent developments of Ad-based cancer gene therapy, including novel engineering techniques of the Ad capsid for efficient tumor targeting, as well as targeting techniques, to restrict transgene expression to cancer cells.

Keywords

adenovirus; cancer; oncolytic virus; review; vector targeting; virotherapy

Gene therapy for cancer represents a novel treatment approach for patients resistant to traditional therapies. The concept of gene therapy is gene delivery as a therapeutic molecular intervention to selectively correct or eradicate defective tissues. Adenoviruses (Ads), in particular are a useful approach for effective gene delivery and expression since they demonstrate several advantages over other vectors, such as the ability to transduce dividing and nondividing cells, the ease of manipulation and the capability to produce high titers of current good manufacturing practice quality. Infection of Ads is mediated by binding of the fiber knob region to the receptor of the target cell, which is the coxsackie–Ad receptor (CAR) for most serotypes, including the most commonly used, serotype 5 (Ad5) (Figure 1). Internalization of the virus is mediated by the interaction of a penton-base Arg–Gly–Asp (RGD) motif and cellular $\alpha_v\beta$ integrins [1], which leads to endocytosis of the virion via clathrin-coated pits. After disassembling of the virus in the endosome, viral DNA is transported to the nucleus and viral genes or transgenes are expressed. Ad DNA is not integrated in the host genome and therefore the risk of mutagenesis is low. Recent reports have found evidence

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regarding secondary receptor interactions, such as heparan sulfate proteoglycans (HSPGs) [2,3]. Recently, it has been suggested that expression of CAR is a rate-limiting factor for infectivity with Ad5. A growing number of studies have demonstrated that expression of CAR on primary human tumor cells is highly variable. This has been reported for ovarian, cervical, prostate, head and neck, and bladder cancer, melanoma, glioma and others [4-14]. Furthermore, loss of CAR expression may be associated with malignant progression and increased tumor aggressiveness [15]. The function of CAR is not fully understood, but it is localized in the tight junctions, which suggests a role in cell adhesions [16]. Despite exciting preclinical data, anticancer molecular therapies have been proposed as a treatment alternative for advanced cancers resistant to conventional treatment modalities. To this end, Ad vectors have been used for a wide variety of gene therapy applications [17]. Unfortunately, the efficacy of gene transfer to solid tumors has been limited and thus there is little evidence supporting significant clinical benefit; only Phase I and II trials have been reported to date [10,18]. The low efficacy of Ad-based gene therapy trials has been attributed to insufficient transduction of tumor cells. Consequently, various approaches have been evaluated to modify or circumvent CAR deficiency, including genetic capsid modifications or retargeting complexes.

Infection with Ads results in a humoral and cellular immune response towards the vector and infected cells. This effect might be potentially beneficial, as it might activate the immune system for recognition of tumor antigens. However, administration of large doses of a viral vector might result in acute toxicity. In general, side effects of Ad gene therapy are rare and mild. However, effective targeting strategies are necessary to further increase target transduction and reduce the risk of unspecific side effects to nontarget tissues.

Genetic targeting approaches

To improve the utility of Ad-based gene transfer vectors in the context of clinically applicable cancer gene therapy, it is essential to increase targeting to tumor cells and/or decrease targeting to the liver. A number of strategies have been developed to alter the tropism of Ad to improve its utility as a gene transfer vector. Increased transduction of low CAR tumor cells can be achieved via genetic retargeting approaches. Ad fiber pseudotyping means alteration of the virus tropism by substituting the receptor-binding proteins (knob/fiber) with those from other serotypes. One such approach is the substitution of Ad5 with a knob from Ad serotype 3 (Ad3). Ad5/3 chimeras have displayed enhanced infectivity in ovarian cancer cells without increasing gene delivery to murine livers [14,19]. Furthermore, Ad5/3 biodistribution, liver toxicity and rate of blood clearance appear to be no different from Ad5 when administered intravenously or intraperitoneally, suggesting an excellent safety profile for Ad5/3 in preclinical tests.

Rigorous structural analysis of the fiber knob domain have led to alternative strategies based on the incorporation of targeting ligands into the Ad knob domain without ablating native CAR-binding. One option is the genetic incorporation of an RGD-4C peptide into the HI loop or the C-terminal end of the Ad5 fiber knob [11,20,21]. This allows Ad-mediated gene transduction through the binding of the RGD peptide to integrins on target cells. A different approach is tumor cell targeting to HSPGs via positively charged polylysine (pK7) motifs [22]. HSPGs are common constituents of cell surfaces and the extracellular matrix, and they are overexpressed in several different cancer types including cervical cancer [23-26]. Double modified vectors containing both RGD and pK7 motifs increase infectivity in different cell types [27,28].

However, systemic administration of tropism-modified adenovirus vectors results mostly in hepatic gene delivery [29,30]. Recent studies have shown that the Ad5 fiber shaft is responsible for liver gene delivery [3,31]. In this regard, strategies to genetically replace the Ad5 fiber shaft can reduce hepatic vector delivery *in vivo*. The length of the fiber plays an important role in viral infectivity and tropism [32-34]. It is well known that Ad5 internalization requires an

additional interaction of RGD motifs on the penton base with α_v integrins on the cell surface [35]. Ad5 vectors with short-shafted fibers do not bind efficiently to α_v integrins. However, *in vivo* vector delivery to the liver seems to occur independently of CAR and α_v integrins, whereas binding to heparan sulfate glycosaminoglycans (HSGs) seems to be the major determinant for hepatic gene transfer [2].

Conditionally replicative adenoviruses

The first generation of Ad vectors were able to infect cancer cells but were unable to replicate within the cell. Unfortunately, most cancer trials with replication defective Ads demonstrated that transduction rates of tumor cells were too low for significant therapeutic efficacy [36-38]. It is likely that strategies to enhance infectivity of nonreplicating vectors could improve results dramatically [5,39,40]. However, for advanced disease with increased tumor masses, tumor penetration is the key to efficacy. A new and powerful approach for increasing transduction of tumor cells is the use of replication-competent oncolytic agents, such as conditionally replicative Ads (CRAds).

Most approaches for the development of CRAds have focused on the genetic engineering of early 1 (E1) genes. The major tasks of E1 proteins are to induce expression of further viral genes, and to orchestrate modifications of cellular gene expression and protein activity that favor viral replication. Two strategies have been used to restrict virus replication to target cells and to spare normal tissue. Genetic complementation-type (type 1) CRAds [41,42], such as Ad524, have a mutation in the immediately early (E1A) or early (E1B) adenoviral region, which is complemented in tumor cells but not in normal cells. In transcomplementation-type (type 2) CRAds, virus replication is controlled via a tumor/tissue-specific promoter [43]. However, in both instances, ectopic liver transduction is the major predicate of Ad vector-induced toxicity.

The prototype of Type 1 CRAds is dl1520 or ONYX-015 [41]. ONYX-015 has two inactivating mutations in the gene that codes for the E1B55k protein. One function of E1B55k is to bind and inactivate the tumor-suppressor protein p53. Thus, the rationale for this strategy is that the virus can only replicate in cells in which p53 or p14^{ARF} is mutated. However, it is apparent that this CRAd does not replicate in tumor cells to a larger degree than in normal cells. This is probably due to the lack of E1B55k functions different from p53 activation. Another type-1 CRAd was derived by mutation of *E1A*. *E1A* mutant CRAds, such as Ad5 Δ 24 or CB016, contain specific mutations in individual domains of the protein [42,44,45]. This strategy aims to ablate S phase induction by the CRAds, which is not required in proliferating tumor cells or in dysplastic cervical cells transformed by human papillomavirus (HPV) viruses.

Type 2 CRAds are designed to contain a tissue-specific promoter (TSP), upstream of the *E1A* gene for specific expression in target cells. Critical for the development of Type 2 CRAds is the availability of promoter fragments with specific activity. To date, the structure–function relationship of promoter regions is not well understood and construction of TSPs is relatively empirical. However, data from preclinical studies are promising.

Tissue specific promoters

For cancer gene therapy, it is rational to choose a TSP that is expressed highly in the tumor but has potentially low activity in the liver. One of the most promising promoters for ovarian cancer is the promoter for secretory leukoprotease inhibitor (SLPI). SLPI is a 11.7-kDa serine protease inhibitor, which is highly expressed in different human carcinomas, including breast, lung, endometrium and ovarian cancer. Gene expression analysis has been used to demonstrate that SLPI is 60-fold upregulated in ovarian carcinomas compared with normal ovarian epithelium [46,47]. In addition, SLPI is expressed minimally in the normal liver [48].

Recently, a chimeric 5/3-fiber-modified SLPI CRAd was shown to have a strong antitumor effect compared with wild-type Ad. Furthermore, the chimeric SLPI CRAd demonstrated very high efficacy in an orthotopic murine model of ovarian cancer.

Another attractive candidate for targeted therapy of ovarian cancer is mesothelin (MSLN), a glycosylphosphatidylinositol-linked cell surface glycoprotein that is overexpressed in different tumor types, such as ovarian cancer, mesotheliomas and several different squamous cell cancers [49,50]. MSLN is not present on normal tissues, except mesothelial cells and is also not shed into the bloodstream in significant amounts. The high tumor specificity of a MSLN-targeted Ad has been demonstrated recently in an ovarian cancer mouse model [51]. Another promising promoter in the context of ovarian cancer gene therapy is cyclooxygenase (COX)-2. Recently, an infectivity-enhanced COX-2 CRAd, which has an RGD-4C motif in the Ad fiber knob region, was shown to have a strong antitumor effect compared with wild-type Ad5.

The promoter of vascular endothelial growth factor (VEGF) is an effective target molecule for the treatment of non-small cell lung cancer (NSCLC). VEGF is one of the most important inducers of angiogenesis and is upregulated in various cancer types, including carcinoma of the cervix uteri [52-54]. Midkine is a heparin-binding growth factor identified as a retinoic acid-responsive gene. Midkine has been reported to be over-expressed in a wide number of malignant tumors. The Midkine promoter has been used successfully for vector gene delivery [55,56] and CRAD targeting approaches [57]. The promoters of the CXC chemokine CXCR4, as well as Survivin, exhibit an optimal profile of activity and specificity in different tumor types, such as breast cancer. It was demonstrated recently that CXCR4 expression is upregulated in breast cancer cells but is not detectable in normal mammary epithelial cells [58]. Survivin is a member of the inhibitor of apoptosis (IAP) protein family and the expression levels of the *Survivin* gene have been shown to be of clinical value in the detection of human gliomas [59]. Both genes expressing CXCR4 and Survivin play a major role in progression and metastasis of various tumor types [60,61] and are therefore rational targets for cancer therapies. *Survivin* has been shown to be overexpressed in cervical intraepithelial neoplasias (CINs) and squamous cell carcinomas (SCC) of the uterine cervix [62]. Selected tumor-specific promoters are summarized in Table 1.

Conclusion

Ad-based gene therapy vectors are the most widely used platform for gene delivery. Their potential utility for cancer treatment is high, although results of clinical trials with nonreplicative first generation Ads were disappointing. Replicating Ad vectors offer high promise as agents for cancer treatment and have been used safely in human clinical trials (Table 2) [63,64]. In addition, molecular modifications, such as genetic targeting approaches or chimeric-fibers, have substantially improved the efficiency of these second-generation virotherapeutics in preclinical trials. However, the outcome of clinical trials using tropism-modified CRAds will be of special interest. Until today, preclinical efficacy and safety data are preliminary owing to the limitations of currently available animal models. To address these shortcomings, we have developed a novel *in vitro* toxicity assay based on precision cut human liver slices. Preliminary data demonstrate the effect of transcriptional targeting to restrict virus replication to target cells.

Executive summary

Adenovirus-based gene therapy is a promising approach for cancer therapy

- Viral gene delivery has been optimized by evolution.

- Advantages of adenovirus (Ad) vectors include the ability to transduce dividing and nondividing cells, the ease of manipulation and the capability of producing high titers.
- A disadvantage of Ad vectors is natural liver tropism, which can lead to severe side effects after systemic vector delivery.

The low expression of the primary Ad receptor coxsackie-Ad receptor on tumor cells is the rate limiting factor for cancer gene therapy

- The coxsackie-Ad receptor (CAR) is the primary receptor of most Ad serotypes, including the most commonly used serotype 5 receptor.
- Unfortunately, CAR expression is highly variable and often low on the surface of most tumor cells.

Effective tumor targeting can be achieved via genetic retargeting strategies

- Various strategies have been evaluated to modify Ad tropism in order to circumvent CAR deficiency.
- Ad fiber pseudotyping is an approach to substitute receptor-binding proteins to alter the virus tropism.
- Incorporation of targeting ligands into the Ad knob domain can increase tumor transduction without ablating native CAR-binding.

Specific tumor targeting can be achieved via tumor-specific targeting strategies

- Targeting of viral vectors to cellular target structures that are highly and exclusively expressed on tumor cells.
- Tissue-specific promoters (TSPs) upstream of the viral *E1A* gene allow specific transduction of target cells.
- It is rational to choose a TSP that is highly expressed in the tumor but has low activity in healthy organs, such as the liver.

Virus replication is needed to face advanced solid tumor masses

- Conditionally replicating Ads (CRAds) take advantage of tumor-specific features that allow preferential replication in tumor cells.
- Virus replication causes oncolysis of the cell, resulting in the release of the virus progeny and subsequent infection of neighboring cells.
- Replication-competent viruses can be armed with therapeutic transgenes, such as suicide genes or antiangiogenic moieties.

Radio-chemo-gene therapy

- The lack of overlapping toxicities allows the development of multimodality treatment approaches, which offer great promise in the near future.

Future perspective

Gene therapy strategies have the potential to increase the survival of cancer patients if gene therapy is included in a multimodality treatment approach. The major challenge is the development of effective gene therapy vectors since the vectors available currently mostly result in insufficient gene transfer. Targeting of viral vectors to cellular target structures that are highly and exclusively expressed on tumor cells is a promising approach for the next 5–10

years. Such modified vectors would be able to effectively penetrate solid tumor masses. The current generation of replication-competent Ads demonstrates superior tumor penetration and oncolysis when compared with first generation viruses. In the near future, replication competent viruses will be armed with therapeutic transgenes, such as suicide genes, or cytokines. Since solid tumors can only be destroyed step by step, successful gene therapy approaches will require several rounds of virus administration, whose efficacy may be inhibited by neutralizing antibodies. Strategies to reduce the effect of neutralizing antibodies, which might limit the efficacy of a virotherapy, include alternating related viruses with different capsids or cotreatment with immunosuppressive drugs for temporary abrogation of neutralizing antibodies.

Recent studies have demonstrated the synergistic effect of combination therapies consisting of radio- and/or chemo-therapy and virotherapy. The lack of overlapping toxicities allows the development of multimodality treatment approaches, which offer great promise in the near future. The rapidly growing number of molecular targets will allow us to design individualized Ad vectors according to individual tumor characteristics.

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Figure 1.
Structure of adenovirus depicting major structural components of a wild-type adenovirus capsid.
Adenovirus capsids contain an up to 36-kilobase double-stranded DNA genome. Selected tumor-specific promoters for gynecological cancer in serotype 5 (Ad5).

Table 1

Selected tumor-specific promoters for gynecological cancer in Ad5-based gene therapy vectors.

Author	Tumor-specific promoter	Disease application	Transgene
Kanerva <i>et al.</i> (2004)	COX-2	Ovarian cancer	CRAD
Casado <i>et al.</i> (2001)			HSVtk
Breidenbach <i>et al.</i> (2004)	Mesothelin	Ovarian cancer	luciferase
Rein <i>et al.</i> (2005)	SLPI	Ovarian cancer	CRAD
Barker <i>et al.</i> (2003)			HSVtk
			Luciferase
Zhu <i>et al.</i> (2004)	CXCR4	Breast cancer	Luciferase
Zhu <i>et al.</i> (2004)	Survivin	Cervical cancer	Luciferase

Ad: Adenovirus; Ad5: Serotype 5; COX: Cyclooxygenase; CRAd: Conditionally replicative Ads; CXCR: Chemokine (CXC) motif receptor; HSVtk: Herpes simplex virus thymidine kinase; SLPI: Secretory leukoprotease inhibitor.

Table 2

Outcome of clinical Phase 1/2 trials with replication-competent Ads.

Replication-competent Ads	Disease	Outcome	Refs
ONYX-015	Head and neck cancer (n = 24)	Rapid tumor progression in most patients	[63]
ONYX-015	Head and neck cancer (n = 37)	Combination of gene therapy and chemotherapy with cisplatin and 5-FU, reasonable response rates	[64]
ONYX-015	Pancreatic cancer (n = 23)	No responses after intratumoral virus application	[65]
ONYX-015	Metastatic liver cancer (n = 27)	Moderate responses with a combination of gene therapy and chemotherapy with 5-FU and leucovorin	[66]
ONYX-015	Metastatic colon cancer (n = 18)	Stable disease in 7 patients, moderate side effects	[67]
ONYX-015	Pancreatic cancer (n = 21)	Stable disease in 6 patients, combination of gene therapy and gemcitabine	[68]
CG7060	Prostate cancer (n = 20)	Intratumoral treatment, decrease of PSA levels	[69]
Ad5-CD/TKrep	Prostate cancer (n = 15)	Combination of radiotherapy and double-suicide gene therapy	[70]

Ad: Adenovirus; Ad5: Serotype 5; CD: Cytosine deaminase; CG7060: A PSA-specific, replication-competent adenovirus; FU: Fluorouracil; ONYX-015: An oncolytic adenovirus; PSA: Prostate-specific antigen; Ad5-CD/TKrep: Replication competent Ad.