Targeting Adenoviral Entry to Enhance Oncolytic Antitumor Response

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Abstract: Conditionally replicative adenoviruses represent an innovative group of anticancer agents designed to destroy these cells by replication and lysis. A major problem associated with of the use of adenoviral vectors in gene therapy is its high liver uptake and lack of tumor selectivity upon systemic administration. To improve the efficacy of CRAds as anticancer agents, their infection efficiency on CAR-deficient tumor cells could be enhanced their by redirecting viral entry *via* a CAR-independent pathway. To redirect the entry pathway of adenoviruses and enhance their infectivity and specificity, two general strategies are being used. In the first strategy, the adenovirus genome is changed to alter the binding specificity of the viral capsid. In the second strategy, a two-component targeted adenovirus is created by binding of proteins with specific affinity for cancer cells onto the viral capsid. Despite effective targeting and tumor eradication *in vitro* and in mouse models, the results from systemic administration of targeted CrAds is limited. In addition, clinical effects of CrAds are disappointing up till now. Therefore, combination therapies in which targeted CrAds are combined with other types of therapy are being investigated.

Keywords: Conditionally replicative adenoviruses, targeting, peptides, bispecific single-chain antibodies.

INTRODUCTION

Cancer Gene Therapy

Gene therapy involves the delivery of genes to specific cells of interest in order to treat a disease. In this manner, gene therapy can theoretically be used to deliver toxin or corrective genes to tumor cells specifically, which would be an innovative and promising treatment strategy for cancer. This is limited in practice however, due to a low efficiency and specificity of gene delivery to target cells. Improved delivery vehicles are therefore required for a specific gene delivery and expression in target cells, without harming healthy cells.

Delivery by viral vectors is the most common systemic delivery strategy currently being investigated in gene therapy. Viral vectors are used in almost 70% of clinical trials in gene therapy, 24.7% being adenoviral vectors [1]. Advantages of the adenovirus are its high expression of transgenes, its large DNA payload capacity, its high stability and low pathogenicity *in vivo* and its ability to infect quiescent as well as dividing cells. Conditionally replicative adenoviruses [CRAds] represent a novel class of anticancer agents designed to selectively replicate in tumor cells and to destroy these cells by inducing lysis [2, 3].

ADENOVIRAL CELL ENTRY BIOLOGY

The basic adenoviral structure is an icosahedron capsid formed by hexon proteins. A penton assembly is attached to the vertices, formed by the penton base and fiber proteins protruding from it [4].

Human Ad serotypes of subgroup B [serotypes 3, 7, 16, 21, 50, 11, 14, 34, 35] use CD46 as a primary attachment receptor [5]. CD46 is a member of a family of proteins that regulate complement activation and is expressed on all human cells with the exception of erythrocytes [6].

Binding of adenoviruses serotypes of subgroup A, D, E and F is mediated by the tethering receptor CAR [coxsackie and adenovirus receptor] on the cell surface, comprising the first step in cell entry of the virus [5, 7, 8]. The immunoglobulin-like D1 domain of this CAR receptor is able to bind to the carboxyterminal knob domain of serotype 5 fiber [9], following cell-surface $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin mediated virus internalization through the arginine-glycine aspartic acid [RGD] sequence of the adenoviral penton base protein [10]. The viral particle is internalized into a clathrin-coated endosome, from which it can be released into the cytoplasm through acidification of the endosome [11]. After release the particle is being translocated to the nucleus, following replication of the virus.

A major drawback of the use of adenoviral vectors in gene therapy is its high liver tropism and lack of tumor tropism upon systemic delivery. Over 90% of intravenously administered adenovirus is rapidly taken up by the liver Kupffer cells through the scavenger receptor [12], giving rise to hepatotoxicity, a reduction in the viral transduction of target cells and thus decreased therapeutic efficiency.

Unfortunately, primary human cancers express low levels of CAR and are refractory to adenovirus infection [13-18]. In particular, high-grade undifferentiated tumors exhibited decreased CAR expression [19, 20]. Furthermore, primary

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tumors revealed heterogeneous CAR expression, sometimes with a focal distribution pattern [14, 20]. Thus, lack of CAR expression in primary tumors or tumor regions may hinder CRAd-based therapies. Therefore, abolishment of CAR mediated adenoviral cell entry and tumor retargeting has received much attention in studies focusing on improvement of adenoviral targeting specificity.

CONDITIONALLY REPLICATIVE ADENOVIRUSES [CRAds]

Conditionally replicative adenoviruses [CRAds] represent a novel class of anticancer agents designed to selectively replicate in tumor cells and to destroy these cells by inducing lysis [2, 3]. The first CRAd developed, is known as ONYX-015 or dl1520. It carries a deletion in the E1B-55kDa coding region that introduces selectivity for tumors with dysfunctional p53 [21]. The normal function of the E1B 55 kDa protein is to bind to and inactivate p53 protein in infected cells. Because Onyx-015 lacks this protein, it was originally thought to replicate in a p53-dependent manner. Later it was shown that tumor selectivity of this CrAd was because of an altered nuclear RNA export pathways [22]. This approach has potential advantages over the traditional therapies of chemotherapy and radiotherapy because of the possibility of targeting tumors at a molecular level while leaving normal tissue relatively unaffected [23].

Promising preclinical results led to rapid translation to clinical trials for head and neck cancer [24, 25], pancreas carcinomas [26], and malignant glioma [27]. ONYX-015 was infused intravenously in patients with advanced carcinoma metastatic to the lung. No dose-limiting toxicity was identified [23, 25]. Clinical efficacy of this virus however was low [28, 29]. In 2006 the first intratumoral administrated oncolytic adenovirus was clinically approved to treat head-and-neck cancer patients in a combination with chemotherapeutic treatment [30].

Other types of CRAd include the Ad-delta-24 or dl922-947 which carries a partial deletion in the CR2 domain of the adenoviral E1A gene that abrogates the binding of E1A to pRb for the treatment of glioma [3, 31-33]. Epstein-Barr virus specific replication in nasopharyngeal carcinoma [34], human papillomavirus in anogenital cancers [35], tyrosinase to target melanoma [36, 37], CXCR4 to target ovarian cancer [38] and PSA for prostate cancer [39] for a review see [40, 41] and this issue.

TARGETING STRATEGIES FOR ONCOLYTIC ADENOVIRUS

A rational approach to improve the efficacy of CRAds as anticancer agents could be to enhance their infection efficiency on CAR-deficient tumor cells by redirecting viral entry *via* a CAR-independent pathway. This should augment the infection efficiency during replication and lateral spread in a CAR-deficient or heterogeneous tumor. Currently, two general strategies are being considered to redirect the entry pathway of adenoviruses in order to enhance their infectivity and specificity. In the first strategy, the adenovirus genome is modified to alter the binding specificity of the viral capsid [42]. In the second strategy, a two-component targeted adenovirus is constructed by coupling proteins with specific binding affinity onto the viral capsid [14, 16, 18, 43-48].

PSEUDOTYPED ADENOVIRUSES

In the context of CRAds, the modification of the adenovirus genome to alter the binding specificity of the viral capsid appears most appropriate because the targeting specificity is made inherent to the viral genome and thus maintained upon replication. Many human Ad serotypes of subgroup A, D, E and F recognize CAR whereas CD46 was identified as a cellular receptor for the majority of subgroup B [serotypes 3, 7, 16, 21, 50, 11, 14, 34, 35] Ads [5]. Thus exchanging the capsid proteins from one serotype with another should confer a different cell specificity.

The utility of this strategy was confirmed by oncolytic replication of an adenovirus with a chimeric Ad3/Ad5 fiber protein to target infection *via* the putative Ad3 receptor in renal cell carcinoma models resistant to Ad5 infection [49] and head and neck cancer. [26, 50, 51] The cyclooxygenase-2 [Cox-2L] promoter was found to be the most advantageous in pancreatic cancer cell lines. An Ad5/Ad3 CRAd with replication of the virus controlled by the Cox-2L promoter was found to safely exhibit replication within a tumor in this model and was found to suppress tumor growth after systemic delivery [52].

Stoff-Khalili MA *et al*. [53] constructed an oncolytic adenovirus using the human CXCR4 gene promoter in an 5/3 fiber-modified CRAd. The oncolytic activity of this virus was studied in breast cancer cell lines, primary breast cancer and human liver tissue slices from patients, and in a xenograft breast cancer mouse model. The pseudotyped CRAd agent showed improved replication and killing in breast cancer cells *in vitro* and *in vivo* with a remarkable specificity profile that was strongly attenuated in non-breast cancer cells, as well as in normal human breast and liver tissues.

Guse K [54] took this approach one step further and constructed Ad5/3-9HIF-Delta24-VEGFR-1-Ig, an oncolytic pseudotyped adenovirus secreting soluble VEGF receptor to block angiogenesis. In an intraperitoneally disseminated kidney cancer model, significantly enhanced survival was observed with the Ad5/3-9HIF-Delta24-VEGFR-1-Ig when compared with control viruses.

Hoffmann D *et al.* [55] used Ad5/35 fiber chimeric adenovirusses with vector binding redirected to the Ad35 receptor and replication under the control of the GFAP/Ki67 or E2F-1/COX-2 promoters. The native Ad5 was compared to the chimeric Ad5/35 fiber for their antineoplastic activity in a subcutaneous and intracranial glioblastoma xenograft model. Animals treated with the Ad5/35-based vectors showed significantly smaller tumors and longer survival than those treated with the homologous Ad5 vectors.

INCORPORATION OF TARGETING PEPTIDES

Although useful to demonstrate the benefit of targeting, application of pseudotyped adenoviruses is limited by the targeting repertoire of naturally occurring adenovirus serotypes. As an alternative, CRAd tropism was expanded

through incorporation of defined targeting peptides in the Ad5 fiber knob.

Insertion of the RGD [Arg-Gly-Asp] sequence, known to interact with v integrins, into the Ad fiber knob improved oncolytic potency on cancer cells *in vitro* and *in vivo* [56, 57]. It was demonstrated that combining this strategy with the Ad-delta-24 CRAd led to strong oncolytic effects in lung adenocarcinoma, prostate cancer, glioma and ovarian cancer cells [58-63]. To further improve tumor selectivity Cascallo *et al.* [64] combined the E2F promoter with the the Rb selectivity of delta 24 deletion in the E1 gene of the adenovirus in an RGD targeted virus. A single intravenous administration in three preclinical models in mice showed significant tumor growth inhibition [65].

Ranki *et al.* [66] generated a novel p16/retinoblastoma pathway-dependent CRAd, Ad5.pK7-Delta24, with a polylysine motif [Lys 7] in the fiber C-terminus, enabling CAR-independent binding to heparan sulfate proteoglycans [HSPG]. Ad5.pK7-Delta24 mediated effective oncolysis of breast cancer cell lines *in vitro*. A therapeutic benefit was seen following both intratumoral and intravenous delivery in an orthotopic model of advanced hormone refractory breast cancer. Piao Y *et al*. [67] described a Delta-24 adenovirus which was retargeted through the abrogation of CAR binding [Y477A mutation in the adenoviral fiber protein] and insertion of an epidermal growth factor receptor[EGFR]-specific binding peptide in the HI loop of the fiber protein. Treatment with this CrAd prolonged the survival of animals with intracranial xenografts derived from glioma cells. Similarly, a survivin driven oncolytic adenovirus targeted to heparan sulfate, was shown to be superior to Ad targeted to CD46 or RGD in glioma [68].

Nishimoto T *et al.* [69] evaluated a targeted adenovirus derived from a random peptide library displayed on an adenoviral fiber knob. In this approach one may overcome the limitation that many cell type-specific ligands are not compatible with adenovirus fiber assembly. Replication-competent adenovirus displaying selected peptides showed higher oncolytic potency in several pancreatic cancer cell lines compared with the untargeted adenovirus. Intratumoral injection of the peptide-targeted CrAd significantly suppressed the growth of subcutaneous tumors.

Thus, the potential of peptide-targeted CRAds for the treatment of cancer is clearly established. A major obstacle for wider application of fiber-modified CRAds with other recognition specificities is, however, that the adenovirus fiber appears to impose not yet fully understood structural demands on the incorporated ligand [70-72]. In general, only relatively small targeting ligands have been successfully rescued into native Ad fiber; presumably, incorporation of larger motifs destabilizes fiber structure and prevents trimerization. As an alternative, another Ad capsid protein, pIX, can be used to present large fusion proteins on the outside of the Ad capsid [73]. For example, Meulenbroek *et al*. [74] generated a pIX–green fluorescent protein [GFP] fusion protein and showed that it is efficiently incorporated into the Ad capsid. pIX . In addition, the adenovirus fiber shaft or hexon has been modified to contain an RGD peptide motiv [75, 76]. In order to allow the incorporation of larger peptides, Belousova *et al*. [70] replaced the Ad fiber with the trimerization domain of the phage T4 fibritin, and showed that the fibritin could be fused to the human CD40 ligand. CD40 belongs to the tumor necrosis factor [TNF] receptor superfamily and is highly expressed on myelomas, dendritic cells and tumor cells cells.

Thus larger binding ligands may be incorporated into the Adenovirus capsid by incorporation into either pIX or a modified fiber protein, however, thusfar these have not been used in the context of replication competent Ads.

ADAPTER TARGETED CRAd

The repertoire of genetically targeted CRAds could potentially be expanded tremendously if more complex, high-affinity ligands such as antibodies could be used. However, such molecules have so far not been successfully incorporated as a stable component of the adenovirus capsid. In addition, the nuclear assembly of adenoviruses may preclude correct post-translational modification of protein moieties that are normally processed through the secretory pathway. For these reasons, complex binding ligands, including antibodies, have so far only been successfully employed in two-component targeting strategies, where they were bound to the adenovirus fiber indirectly *via* a second protein moiety [14, 16, 18, 43-47, 77]. This leads to an increased target specificity by ablating CAR-mediated entry, since the viral fiber knob is blocked by the adaptor protein. A disadvantage of the bridging strategy employed by using bispecific adapter proteins in the retargeting of adenoviral vectors to tumor cells specifically lies in the fact that the targeting ligand has to be genetically incorporation into the adenoviral vector genome to avoid loss upon viral replication.

GENOMIC ADAPTER TARGETED CRAd

A single component targeted adenovirus was developed by van Beusechem *et al.* [78], who constructed a new type of targeted CRAd that carries an expression cassette for a secreted antibody targeting moiety in its genome. The targeting moiety was the bispecific single-chain [scFv] antibody 425-S11 consisting of the anti-epidermal growth factor receptor [EGFR] scFv 425 and anti-adenovirus fiber knob scFv S11 that targets adenovirus entry *via* EGFR [47, 79]. In contrast to CAR, EGFR is commonly overexpressed in many tumor types [80]. The novel EGFR-targeted CRAd produced its own bispecific scFv targeting moiety, while replicating in CAR-deficient cancer cells, providing its progeny with CAR-independent infectivity. As a result, the EGFR-targeted CRAd exhibited an improved oncolytic potency on CAR-deficient cancer cells, primary brain tumor specimens, and 3-D tumor spheroids *in vitro*.

Carette *et al*. [24] constructed and characterized an oncolytic adenovirus, carrying mutated capsid proteins to abolish the promiscuous adenovirus native tropism and encoding the same EGFR-targeted bispecific adapter molecule. The new virus displayed a highly selective targeting profile, with reduced infection of EGFR-negative cells and efficient killing of EGFR-positive cancer cells including primary EGFR-positive osteosarcoma cells that are refractory to infection by

conventional adenoviruses. Hence, this CRAd combines the therapeutic gains of targeting and oncolytic replication, in a stable single-component genetic anticancer medicine, with the flexibility of a two-component targeting strategy.

DETARGETING ADENOVIRUS FROM NORMAL TISSUE

Proper targeting of CRAds to tumor cells should fulfill two requirements; i.e., the virus should be engineered to transduce the cells of interest with high efficiency and its natural tropism towards nontargeted tissues should be abolished. To achieve strict targeting, the native tropism of the CRAd should be abolished. This is especially important for systemic delivery of CRAds because the vast majority of intravenously injected adenovirus is sequestered in the liver [12].

The presence of CAR on erythrocytes leads to a prolonged *in vivo* blood half-life and significantly reduced liver infection when a CAR-tropic Ad was injected intravenously [81]. In addition, integrins appear to mediate Ad binding to platelets. Platelets bound to Ad displaying an RGD ligand in the fiber knob more efficiently than unmodified Ad [82].

Recently, it was described that in addition to the CAR-integrin pathway, blood-borne adenovirus infects hepatocytes through an indirect pathway that involves blood coagulation factors [83-85] which may bridge to cellular heparan sulfate glycosaminoglycans [HSG]. The HSG putative binding site KKTK of the Ad5 fiber shaft domain has been shown to be involved in Ad5 liver transduction in mice, rats and non-human primates [86]. Liu *et al*. [87] showed that preinjection of snake venom factor X-binding protein [X-bp] reduces hepatocyte transduction and increases the circulation time in blood of an intravenously injected, fiber-chimeric Ad5/35 vector. X-bp pretreatment resulted in improved Ad5/35 transduction of liver metastases and increased the antitumor efficacy of an Ad5/35-based oncolytic adenovirus.

Thus, detargeting adenovirus from normal tissue upon intravenous injection may require ablation of CAR, αv integrin- or heparan sulfate glycosaminoglycans binding sites. Mutations that abolish CAR- and integrin-binding in the genome should produce a CRAd lacking CAR and αv integrin-binding sites capable of producing targeted progeny upon replication in cancer cells [24]. Surprisingly, CAR and integrin double binding-ablated vectors had no effect on hepatocyte transduction in animal studies. However, shaft mutation that ablated HSG binding on the background of a normal capsid was sufficient to abrogate liver transduction *in vivo* [75].

CONCLUSION

In summary, conditionally replicating adenovirusses or oncolytic adenovirusses may be selectively targeted to cancer cells by the direct incorporation of specific peptides or by the expression of a selective adaptor molecule. The ultimate goal of these targeted viruses is to allow systemic administration to treat disseminated disease. Despite effective targeting and tumor eradication *in vitro* and in mouse models, the results from systemic administration of targeted CrAds in animal models are limited. In addition, clinical effects of CrAds are disappointing up till now. Therefore, combination therapies in which targeted CrAds are combined with radiation [88], chemotherapy [89, 90] or therapeutic genes such as TNF-related apoptosis-inducing ligand [TRAIL] [91, 92] or an antiangiogenic protein; vascular endothelial growth factor receptor [93] are investigated. These combination therapies are most likely to produce clinical effects.

ABBREVIATIONS

CAR	=	Coxsackie and adenovirus receptor
EGFR	=	Epidermal growth factor receptor
HIF	=	Hypoxia-inducible factor
KDa	=	Kilo Dalton
Rb	=	Retinoblastoma
VEGF	=	Vascular epithelium growth factor
CrAd	=	Conditionally replicating adenovirus
RGD	=	Arginine-glycine-aspartic acid
ScFv	=	Single-chain variable fragment
TNF	=	The tumor necrosis factor

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