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SYMPOSIUM PAPER

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Targeted and shielded adenovectors for cancer therapy

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Abstract Conditionally replicative adenovirus (CRAd) vectors are novel vectors with utility as virotherapy agents for alternative cancer therapies. These vectors have already established a broad safety record in humans and overcome some of the limitations of non-replicative adenovirus (Ad) vectors. In addition, one potential problem with these vectors, attainment of tumor or tissue selectivity has widely been addressed. However, two confounding problems limiting efficacy of these drug candidates remains. The paucity of the native Ad receptor on tumor tissues, and host humoral response due to pre-existing titers of neutralizing antibodies against the vector itself in humans have been highlighted in the clinical context. The well-characterized CRAd, AdΔ24-RGD, is infectivity enhanced, thus overcoming the lack of coxsackievirus and adenovirus receptor (CAR), and this agent is already rapidly progressing towards clinical translation. However, the perceived host humoral response potentially will limit gains seen from the infectivity enhancement and therefore a strategy to blunt immunity against the vector is required. On the basis of this caveat a novel strategy, termed shielding, has been developed in which the genetic modification of a virion capsid protein would provide uniformly shielded Ad vectors. The identification of the pIX capsid protein as an ideal locale for genetic incorporation of shielding ligands to conceal the Ad vector from pre-existing neutralizing antibodies is a major progression in the development of shielded CRAds. Preliminary data utilizing an Ad vector with HSV-TK fused to the pIX protein indicates that a shield against neutralizing antibodies can be achieved. The utility of various proteins as shielding molecules is currently being addressed. The creation of AdΔ24S-RGD, an infectivity enhanced and shielded Ad vector will provide the next step in the development of clinically and commercially feasible CRAds that can be dosed multiple times for maximum effectiveness in the fight against cancers in humans.

Keywords Adenovirus · Vector · Targeted · Shielded · Cancer

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Conditionally replicative adenoviruses as virotherapy agents for cancer gene therapy

Virotherapy, the use of replicative viruses, is a highly attractive approach, and an alternate approach to standard cancer therapies, including gene therapies. Virotherapy exploits the lytic property of virus replication to kill tumor cells, and thus the self-amplification of the virus allows lateral spread in the tumor and greater tumor cell death from an initial infection of only a few cells (Fig. 1). While initial attempts at virotherapy were abandoned due to toxicity and inefficacy, decades ago [1], this approach has re-emerged in pursuit of the problem of limited tumor transduction experienced with alternate cancer gene therapy strategies [2, 3]. Virotherapy now has greater promise due to better understanding of virus biology and the ability to genetically modify viruses and hence, this knowledge allows researchers to design viruses to replicate in and kill tumor cells specifically.

Ad is a highly desirable vector for utilization in virotherapy approaches, as this virus has many attractive features such as low pathogenicity for humans, lack of

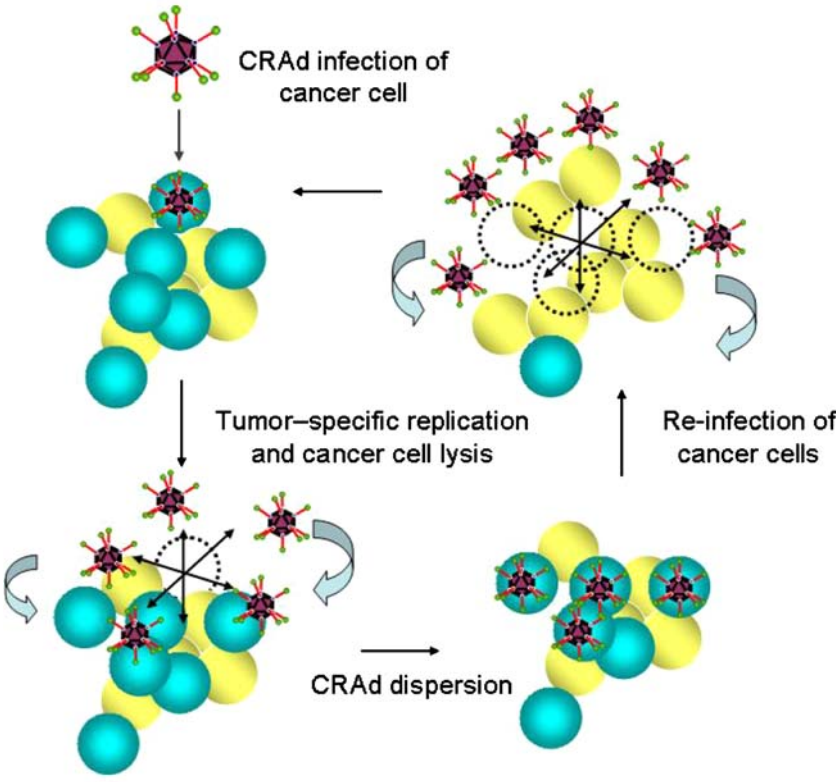

Fig. 1 Oncolytic viral spread

integration in host cell genome and these viruses can be grown to high titers. In addition, they have unique utility for in vivo application due to their high efficacy compared with other approaches [4, 5]. However, three potential limitations may affect the efficacy of the virotherapy approach with adenoviral vectors: (1) Ad does not have natural predilection to replicate in tumor cells, (2) Ad does not efficiently transduce some clinically relevant cancer cells, and (3) Ad cannot be efficiently multi-dosed because of the defense mechanisms of the human body against the vector. Solutions and technological advances to overcome these problems are described below.

Approaches to attain selectivity of replication in CRAd vectors

In the first instance, Ad vectors can be rendered specific for tumor replication through two divergent pathways, selective replication achieved by the regulation of viral genes with tumor-specific promoters (reviewed in depth [6]) and selective replication achieved in theory by the deletion of viral functions dispensable in tumor cells. This second approach to selective replication was pioneered through the use of a mutant Ad (*dl*1520, also known as ONYX-015) that is deleted in the Ad E1B-55 kD protein, which normally binds to and inactivates p53. While such a modification was hypothesized to make the virus (ONYX-015) replicate only in p53-defective cells [7] (the case in 50% of human tumors), this principle has been questioned [8–10]. Furthermore, the replication of this virus was severely hampered compared to wild type virus probably due to the late virus mRNA transcription function of the missing E1B-55 kD protein [10]. The progression of this approach, through second generation conditionally replicative adenoviruses (CRAds) with improved tumor selectivity, has continued despite or perhaps because of the drawbacks realized with ONYX-015 (reviewed [11]).

Of note is the Δ24 Ad with a 24 base pair deletion in the E1A gene domain interacting with the retinoblastoma (Rb) protein which was incorporated into a CRAd and developed independently by two groups [12, 13]. AdΔ24 was shown to be effective in gliomas [12], while its counterpart, dl922–947, was shown to be effective in a range of tumor types [13]. However, there is concern that the therapeutic index actually comes from reduced replication potential within non-dividing/slow growing cells (such as normal cells) versus normal replication within fast growing cells, and hence this mechanism is not fully tumor specific [14] and further modifications were included to improve tumor specificity. Therefore one should perhaps be guarded about using the term tumor specific replication with respect to these CRAds. Regardless of the terminology used many clinical trials have demonstrated safety but limited efficacy, in particular with ONYX-015 (reviewed [11] and [15]) and therefore highlighted the additional confounding limitations, the natural tropism of Ad vectors and the humoral response to Ad vectors.

Modification of natural tropism of adenoviral vectors

The paucity of the natural receptor for serotype Ad5 vectors, the coxsackievirus and adenovirus receptor (CAR), on many cancer tissues (e.g. [16–20]) hinders the efficacy of CRAd virotherapy and therefore the utility of Ad vectors would be further enhanced by re-directing their tropism to alternate receptors. The characterization of the adenovirus (Ad) entry pathway has provided an understanding of the means of modifying Ad tropism. Essentially, cellular recognition is mediated through the globular carboxy-terminal "knob" domain of the Ad fiber protein and CAR [21–23] with internalization of the virion by receptor-mediated endocytosis through the interaction of Arg–Gly–Asp (RGD) sequences in the penton base with secondary host cell receptors, integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ [24]. Post-internalization, the virus is localized within the cellular vesicle system, initially in clathrin-coated pits and then in cell endosomes [25]. The virions escape and enter the cytosol due to acidification of the endosomes, which has been hypothesized to occur via a pH-induced conformational change. Essentially this causes an alteration in the hydrophobicity of the adenoviral capsid proteins, specifically penton base, to allow their interaction with the vesicle membrane. Upon capsid disassembly and cytoplasmic transport, the viral DNA localizes to the nuclear pore and is translocated to the nucleus of the host cell [26].

To develop a truly targeted Ad vector, it is necessary to ablate both native viral tropism and to introduce a novel specificity, which will allow infection of the cells of interest via alternative receptors. Adapter molecule-based, two component systems have demonstrated the feasibility of retargeting through various cell surface receptors (reviewed [27]). Ultimately genetic modification of the fiber protein and/or other capsid proteins provides a more rational approach for introducing a novel cell-specific tropism and permit ablation of CAR interaction. A variety of approaches have been undertaken to achieve novel adenoviral tropism (extensively reviewed [27]). Enhanced and expanded tropism, although not necessarily CAR-independent tropism can be achieved in several ways. One option is through the substitution of Ad5 fiber protein with fibers from other Ad serotypes recognizing alternative receptors. Alternatively this can be achieved through the introduction of relatively short peptide ligands into the so-called HI loop and/or the C terminus of the Ad5 fiber protein. In addition, to achieve selective and CAR-independent tropism an approach that has been described as de-knobbing of the fiber can be undertaken, whereby the fiber is re-trimerized by an alternate trimerization motif to knob that permits the inclusion of large, complex ligands into the chimeric fiber (reviewed [27]). The ablation of the native tropism is not relevant for a CRAd that is locally delivered to a solid tumor, which is its present use. However, the increased specificity achieved by targeting will ultimately allow lower and safer doses of Ad vectors to be provided when regional or systemic delivery is contemplated in the future. By administering the vector to the circulation, a vector with molecular targeting has the potential to reach disseminated cancer that neither be injected mechanically nor is too small to be detected. In this way, molecularly targeted vectors hold the promise to expand the types of diseases that can be treated and make the therapeutic applications of Ad vectors safer and more effective.

Development of infectivity enhanced CRAd, AdΔ24-RGD

In the case of the AdΔ24 CRAd vector, tumor cell specificity is achieved through control of the viral replication cycle. Therefore, instead of achieving specificity though targeting the infectivity enhancement approach was used to increase cancer cell transduction globally by the inclusion of a high affinity RGD motif into the HI loop to direct Ad binding to integrins. The RGD-4C motif was described as a 9 amino acid peptide which binds strongly to the Ad secondary receptors, integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ [28, 29] and incorporation of this motif into the HI loop significantly increased Ad-mediated gene transfer to CAR deficient cell types relevant to various human diseases [30–33] and could restore infection efficiency of CAR-binding ablated Ad vector to the level of wild type Ad [34, 35]. Several cancer tissues are rich in the expression of appropriate integrins, e.g. [36, 37], whereas low in expression of CAR (as discussed previously and [38]). It was hypothesized that such targeting combined with replication control would improve selective killing or even enhance tumor killing [39], especially for cancers that are deficient in the primary adenoviral receptor [40]. The combination of viral gene mutation to control cell-specific replication and transductional targeting led to the development of the AdΔ24-RGD CRAd [39]. This vector has since been shown to enhance tumor killing in a variety of tumor models including gliomas, ovarian and cervical cancers [39, 41–45]. These studies thereby demonstrate great promise for the development of CRAds that can achieve safe, selective, and effective tumor eradication. However, it is still perceived that the host humoral response potentially will limit any gains seen from the infectivity enhancement of AdΔ24-RGD.

Alternate strategies to overcome the humoral response to adenovirus vectors

Within the human population, there are high titers of pre-existing neutralizing antibodies against Ad5 and Ad2 serotypes [46] due to the general exposure to Ads, an issue that has also been discussed in the context of CRAds [11]. Clinical trials utilizing ONYX-015 have confirmed a strong immune response in several patients that had a highly suggestive correlation with the ob-

served limited efficacy of the virus [47–49]. Mathematical modeling of oncolytic Ad spread throughout tumor mass has also predicted that the immune response will be limiting to viral clearance of the tumor [50]. In addition, the anticipated humoral response means effective repeat administration of Ad vectors to most tissues is hindered by a strong neutralizing antibody response to the vector. Thus far skeletal muscle was one of the few tissues where repeat Ad vector administration was successfully demonstrated [51]. However, the success of this procedure was highly dependent on the initial dose of Ad used in the experiment and therefore, it is still expected that repeat dosing in humans will be problematic. Therefore, there is a need to develop Ad vectors capable of evading the humoral response and hence improve clinical utility of these vectors.

In an attempt to circumvent this issue Ad vectors of different serotypes, in particular Ad11 and Ad35, to which the human population has a lower prevalence of neutralizing antibodies, are currently being investigated as alternative vectors to those of Ad5 [46, 52–54]. While these vectors have yet to be developed into CRAds, an oncolytic Ad based on the canine serotype 2 has been developed, although primarily for use in canine models [55, 56]. It is also important to note that different Ad serotype vectors have different intrinsic properties. For example, different serotype vectors use different cellular receptors [57] and it has been shown recently that Ad vectors based on serotype B (like Ad35 and Ad11) binding to their cognate receptor CD46 down regulate immune responses [58] which has yet to be fully determined as a desirable attribute. Other strategies to overcome the humoral response have included alternating serotypes of helper-dependent Ad vectors (in which the genome is completely removed) during repeat Ad vector administration [59] or using chimeric Ad vectors expressing capsid proteins from several different adenoviral serotypes [60, 61]. It is also likely that these approaches will not prevent the development of humoral and cellular immune responses when these vectors delivered multiple times. Therefore, the need for alternate means to develop an immune evasive Ad vector for clinical use still remains.

The process of biochemical modification of the capsid has highlighted a potentially useful strategy. In this way, the Ad vector is shielded from the immune response through molecules covering the capsid. Several studies have demonstrated that conjugation of functional PEG to free lysine groups on the adenoviral capsid enables Ad vectors to avoid neutralizing antibodies, in vitro or in vivo or limit the innate responses [62–66]. An alternate polymer methodology described by the group of L. Seymour performs equally well [67, 68]. In addition, slower clearance rates of Ad from the blood have been demonstrated [69, 70]. Transduction of cells can occur even in the presence of Ad-neutralizing antibodies [62, 65, 71, 72], although too much PEG causes the ablation of the Ad receptor (CAR) specificity resulting in low levels of transduction. Even though these shielded Ad vectors represent a major improvement in overcoming Ad vector limitations for clinical translation, problems such as the multi-component system and the randomness of PEGlyation, and thus heterogeneity of composition will confound potency in batch-to-batch production. These issues alone represent significant problems with respect to scale up and regulatory approval, but the most problematic limitation with this method is that the shielding molecule is lost, due to the replicative nature of CRAd, from the progeny vectors. The development of an alternative means to attach shielding proteins in order to create a uniform shielding method which carries through to the progeny, and in which the replication and lytic Ad function in vivo maintained is critical.

pIX as a novel site for genetic incorporation of shielding molecules

An alternative and potentially much simpler method to chemical shielding is genetic shielding. Essentially the shielding protein would be directly fused to a capsid protein and thus allowing direct incorporation into the viral capsid. This genetic fusion would provide a means to overcome the loss of the shield protein on the progeny of the CRAd. The identification of an optimal capsid locale to permit the genetic incorporation of such moieties is pivotal within the development of a genetic shield. The determination that the minor capsid protein, pIX, displays the carboxy terminus on the outside of the capsid [73, 74] has consequently suggested this capsid locale to be a novel candidate for genetic manipulation. Protein pIX functions as a "cement" stabilizing hexon–hexon interactions, although another function, transcriptional regulator, has been suggested (recent reviews on pIX [75, 76]). It is present at 80 locales, in 240 molecules per capsid, allowing for a defined number of shielding molecules to be included into the capsid, and is positioned to allow the shield proteins to potentially cover the immunodominant epitopes of hexons (Fig. 2).

Recent studies have demonstrated the employment of the carboxy terminus of pIX for genetic Ad capsid modification [77]. It is now known that small proteins such as polylysine, and large complex proteins such as green fluorescent protein (GFP), and herpes simplex virus thymidine kinase (HSV-TK) can be successfully incorporated into the C terminus of the pIX capsid protein with retention of their functionality [78–80], thus indicating a versatile range of proteins that can be incorporated.

To date HSV-TK, at 375 amino acids, is the largest protein to have been successfully fused to pIX, with retention of virion capsid incorporation [78]. Therefore, it was deemed rational to utilize this vector to perform preliminary experiments in the investigation of the shielding effect of a large protein fused to pIX. An ELISA methodology was employed to analyze the concept of shielding effects via HSV-TK fused to pIX capsid

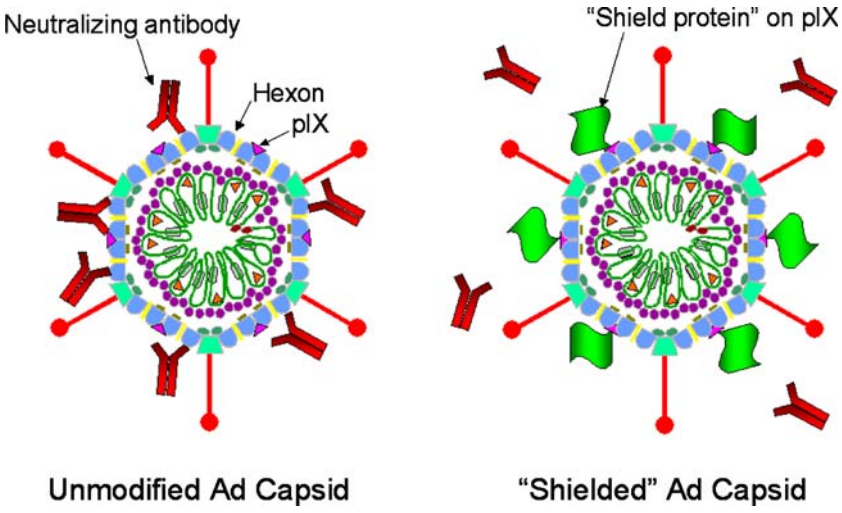

Fig. 2 The adenovirus shielding concept

protein. Serum from naïve mice (pre-immunization) and Ad5 immunized mice (post-immunization) was used as control serum and source of neutralizing antibodies, respectively, with the concept of shielding to be demonstrated through detection of immobilized virions by these antibodies. The preliminary data indicates that significantly fewer antibodies bound the pIX-TK virus from the post-immunized serum than the non-modified virus (approximately 60% less), indicating virion epitopes recognized by the antibodies were reduced due to the presence of TK on pIX. This experiment demonstrates that proteins fused to pIX provide a shielding effect.

However, the shielding protein of choice should embody several important characteristics, namely that it is a self-protein and has sufficient size to cover immunodominant Ad capsid epitopes. A very useful protein, which embodies these properties, is albumin, a large monomeric non-glycosylated polypeptide with wide in vivo distribution, long half-life and lack of substantial immunogenicity [81]. Several proteins have been fused to albumin to enhance circulating half-life and improve stability for therapeutic applications, including, human growth hormone-rHSA (Albutropin) [82], recombinant granulocyte colony stimulating factor-rHSA (Albugranin) [83], and serum albumin-CD4 genetic conjugate [84]. In addition, it has a safe record in clinical practice, e.g., as exemplified by Albuferon™ (albumin-interferon alpha) [85], which has completed phase II clinical trials by Human Genome Sciences [86].

Taken together our data clearly indicate that the ectodomain of pIX is a promising capsid locale for incorporation of heterologous proteins of augmented size and complexity to provide a shielding effect. Current research is underway to determine suitable shielding proteins that perhaps provide a less immunogenic format, i.e., the use of self-molecules, such as albumin rather than foreign molecules such as HSV-TK used in the preliminary study. HSV-TK, of commensurable size to many desired shielding proteins, retains full functionality within the context of the adenoviral capsid when genetically fused to pIX demonstrating structural integrity [78] and suggests large self-molecules may be incorporated into pIX. This body of work indicates the importance of this locale for utilization of an immunological shield for adenoviral vectors and allow for the design of a safe, infectivity-enhanced and shielded CRAd, AdΔ24S-RGD.

Conclusions

Oncolytic Ad provides a novel approach to cancer therapy, known as virotherapy. There are three recognized limitations with respect to CRAd efficacy: (1) tumor/tissue selectivity and hence safety, (2) limited transduction of tumor mass, and (3) the humoral response against the vector, preventing multiple dosing, and possibly spread of vector through the tumor mass. Many laboratories throughout the world are addressing these issues. Our lead product candidate is a well-characterized CRAd, AdΔ24-RGD, a novel infectivity enhanced vector that overcomes the first two limitations, and is the first ever proposed CRAd agent with enhanced infectivity to receive NIH RAC approval for a clinical trial for ovarian cancer and glioma, through the consortium of UAB, MD Anderson Cancer Center and Free University of Amsterdam. The third limitation, that of the humoral response still needs to be studied, and appropriate shielding proteins determined, with research ongoing. However, our data demonstrates that pIX capsid protein is ideal for the genetic incorporation of a shielding protein, due to the positioning of pIX itself within the capsid, and the versatility of ligand size and complexity that can be successfully fused to the C terminus of the pIX capsid protein. Thus, the development of shielded and infectivity-enhanced CRAds will provide an efficient, cost-effective and creative approach to combat not only local disease but also have further application for regional and systemic delivery that could

be potentially effective treatment for a large numbers of cancers including metastatic diseases. We envision the creation of AdΔ24S-RGD as a major progress in the development of clinically and commercially feasible shielded CRAds that can be dosed multiple times for maximum effectiveness against cancers in humans.

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