

Review

Targeted adenoviral vectors

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Abstract

Replication-defective vectors based on human adenovirus serotypes 2 and 5 (Ad2 and Ad5) possess a number of attributes which favor their use as gene delivery vehicles in gene therapy applications. However, the widespread distribution of the primary cellular receptor for Ad, the coxsackievirus and adenovirus receptor (CAR), allows Ad vectors to infect a broad range of cells in the host. Conversely, a number of tissues which represent important targets for gene therapy, such as the airway epithelium and cancer cells, are refractory to Ad infection due a paucity of CAR. Thus, there is a strong rationale for the development of CAR-independent Ad vectors capable of enhanced specificity and efficiency of gene transfer to target cells. In this article we review the approaches which have been employed to generate tropism-modified Ad vectors. These targeting strategies have led to improvements in the safety and efficacy of Ad vectors and have the potential to yield an increased therapeutic benefit in the human clinical context. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The expanding knowledge of the genetic basis of disease has led to the development of novel therapies designed to treat disorders at the molecular level. One such emerging treatment modality is gene therapy, which involves the transfer of genetic material to cells, in order to produce a therapeutic effect. A delivery vehicle, or vector, is employed to transfer the therapeutic gene. One very promising vector is based on human adenovirus (Ad) serotypes 2 and 5 of subgroup C. The use of Ad vectors is advantageous due to a number of positive attributes of the virus [1,2]. First, Ad has the fundamental quality of being able to provide efficient *in vivo* gene transfer to both dividing and quiescent cells as well as possessing high *in vivo* stability. Additionally, ample knowledge about Ad exists since Ad has been extensively studied since its initial description in the early 1950s. Ad vectors can be produced at high titers, which is essential for clinical utility. Finally, Ad vectors are relatively safe; since

Ad is nononcogenic, Ad-related pathology is mostly limited to mild upper respiratory tract infections, and Ad genomes do not integrate, which provides avoidance of germ line gene transfer.

However, Ad vectors are not without their deficiencies. The native tropism of Ad allows Ad vectors to infect a broad range of cells in the host. This promiscuous nature of Ad has led to unsuccessful attempts to physically restrict Ad vectors to a single body compartment in hopes of limiting vector toxicity. Animal studies have shown that Ad vectors do not remain confined to one compartment but are able to disseminate, with resultant toxic effects on distal sites, most notably the liver [3]. This lack of specificity also limits the utility of systemically administered Ad vectors due to the potential toxicity of gene expression in normal tissues and organs, particularly the liver [4], as well as decreasing treatment efficacy by sequestration of Ad vectors in healthy tissues. Conversely, important types of target tissues are refractory to Ad infection. This is highlighted by the poor performance of current Ad vectors in clinical trials, especially in the context of treating the airway epithelium [5] and advanced cancers [6,7]. Taken together, these effects require an escalating dose of vector in order to achieve efficient gene transfer, which in turn increases vector-associated toxicities. Furthermore, Ad vectors evoke a

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potent humoral and cellular immune response, which is enhanced by higher vector doses. Therefore, there is a strong rationale for the development of targeted Ad vectors capable of enhanced specificity and efficiency of gene transfer. In this article we will review strategies by which this can be achieved.

2. Biology of Ad infection

Any strategy to design a targeted Ad vector for cell-specific gene delivery must be based on the structure of the Ad virion and the biology of Ad infection. Adenoviruses (Ad) are nonenveloped viruses containing double-stranded DNA within an icosahedral protein capsid. Two human Ad serotypes (Ad2 and Ad5 of subgroup C) are commonly utilized as vectors for gene therapy. Ad vectors are rendered replication-defective by substitution of the E1 early region of the genome with a therapeutic gene. Ad capsids are comprised of three major protein components—the hexon, penton base, and fiber. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices (Fig. 1). Each penton capsomer consists of five penton base subunits embedded in the capsid that are tightly associated with a trimeric fiber protein which extends outward from the virion. The two components of the penton capsomer, the fiber and penton base, interact with distinct cell surface receptors during the entry of Ad into susceptible cells. In contrast, the hexon has no functional role in the entry of Ad into cells.

Fiber capsid proteins consist of three distinct domains—tail, shaft, and knob. Each domain has distinct functions in host cell infection. The amino (N)-terminal tail domain anchors the fiber to the Ad capsid through association with the penton base [8]. It may also direct the entry of newly synthesized Ad fiber proteins into host cell nuclei through the facilitation of a nuclear localization signal KR λ K (where λ can be any small amino acid residue) [9]. The shaft domain extends away from the virion surface and in Ad2 and Ad5 is comprised of 22 pseudorepeats of 15 amino

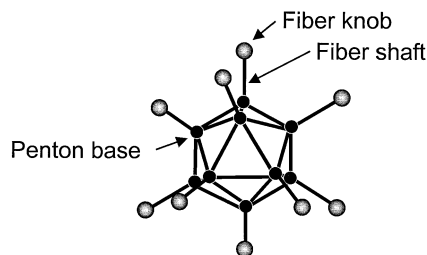


Fig. 1. Schematic diagram of Ad5 virion. The double-stranded DNA genome is packaged within an icosahedral protein capsid. The major structural protein of the capsid is the hexon. Penton capsomers, formed by association of the penton base and fiber, are localized at each of the 12 vertices of the Ad capsid.

acids in a triple- β -spiral conformation [10]. The shaft increases opportunities for host receptor binding by extending the knob away from the virion, and facilitating its interaction with the cell [8]. The trimeric subunits of the carboxyl (C)-terminal knob domain resemble a 3-bladed propeller where two antiparallel β -sheets comprise each subunit. The knob domain is responsible for binding to the host's primary cellular receptor [11,12]. It also initiates and stabilizes the trimeric configuration of the fiber shaft [13,14], which has been shown to be crucial for association of the fiber with the penton base in the assembly of native virions [15].

The primary cellular receptor for the subgroup C adenoviruses Ad2 and Ad5 has been identified as a 46-kDa glycoprotein, named the coxsackievirus and adenovirus receptor, or CAR [16,17]. The extracellular region of this transmembrane protein contains two immunoglobulin-related domains. The N-terminal domain, D1, is related to immunoglobulin V regions and is thought to form a complex with the Ad knob where each trimer of the Ad fiber binds three CAR D1 monomers [18,19]. Importantly, CAR has been shown to be strictly a docking site for the Ad [20,21], providing a high affinity virus-to-host association (Fig. 2). The cytoplasmic and transmembrane domains of CAR are nonessential for host infection. On cells that lack CAR, virus uptake has still been shown to occur [20,21], albeit with low efficiency, which indicates a secondary cellular interaction involved in internalization.

Internalization of Ad2 and Ad5 into host cells is mediated by a secondary interaction between RGD motifs on penton base protein loops and integrins $\alpha_v\beta_3$ or $\alpha_v\beta_5$ [22,23]. It has been hypothesized that Ad particles induce the clustering of α_v integrins that then facilitate the localization of virions into clathrin-coated pits [24]. The phosphorylation of signaling proteins like phosphoinositide-3-OH kinase (PI3K) through the binding of Ad penton bases to α_v integrins has been shown to promote Ad cell entry by facilitating polymerization and reorganization of the host cell's actin cytoskeleton [25]. This provides both a motive force and a route for the trafficking of viral protein to the host cell nucleus [26]. The possibility of the participation of other integrins in Ad internalization has not been ruled out. Low-level infection in α_v integrin-deficient cells suggests that other integrins, such as $\alpha_3\beta_1$ and $\alpha_5\beta_1$, may have a limited degree of involvement [22,27,28]. Without CAR, virus-to-host interaction occurs with a much lower affinity. Therefore, both primary and secondary receptor interactions are necessary for Ad infection.

Clathrin-coated pits of Ad material next undergo endocytosis by means of a cytosolic GTPase, dynamin [24]. Dynamin mediates the constriction of these pits and the budding of coated vesicles during invagination [29,30]. Inside the cell cytoplasm, the virus escapes its vesicle through the lysis of the endosomal membrane. Through microtubule-mediated translocation, the virions enter the nuclear pore complex where the DNA material is released into the

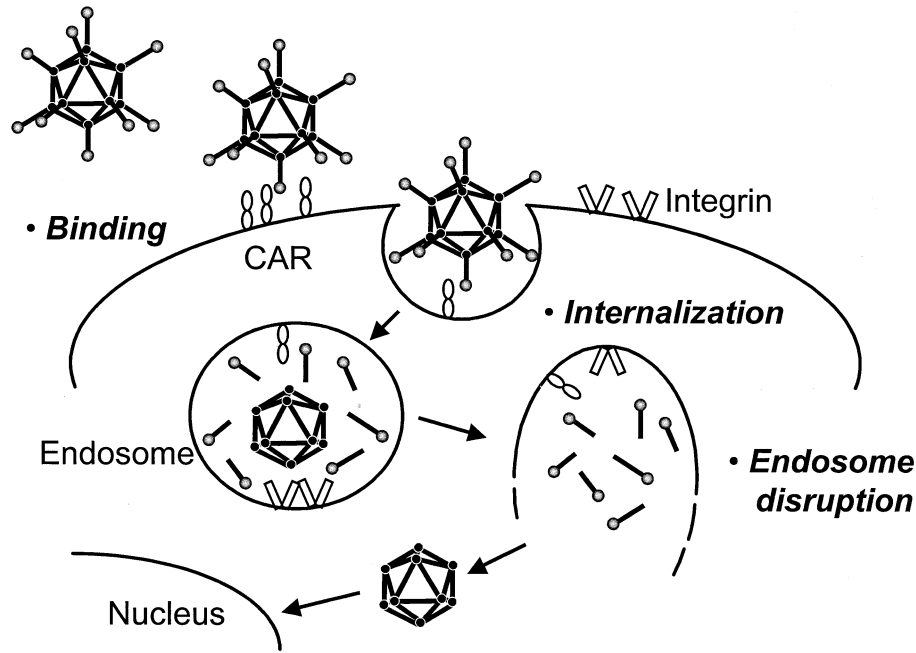


Fig. 2. The pathway of adenoviral infection. The entry of Ad into susceptible cells involves two distinct, sequential steps. The initial high affinity binding of Ad5 to the primary cellular receptor, CAR, occurs via the globular knob domain of the trimeric fiber capsid protein. Subsequent internalization of the virus by receptor-mediated endocytosis is potentiated by the interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base protein with secondary host cell receptors, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. The virion then escapes from the endosome and localizes to the nuclear pore whereupon its genome is translocated to the nucleus.

nucleoplasm and incorporated into the host cell's genome [31,32].

3. Targeting strategies

Therefore, the capability of an Ad vector to infect a cell is based on CAR and integrin expression. Thus, cells expressing CAR and integrins below a threshold level are refractory to Ad infection [33]. Some important cell types resistant to Ad due to a paucity of CAR include mature skeletal muscle [34], endothelial [35,36], smooth muscle [35,36], differentiated airway epithelial [37,38], lymphocytes [20,36,39–41], fibroblasts [35,36,42], hematopoietic cells [43], and monocyte-derived dendritic cells (DCs) [44]. It has also been observed that primary tumor cells are refractory to Ad infection due to a deficiency of CAR [45–49]. These findings therefore explain the poor results from clinical trials mentioned earlier [5–7]. Coupled to this is the problem of sequestration of the vector by CAR-expressing nontarget tissues.

Based on these observations, strategies to modify Ad tropism have focused on alterations to the fiber component of the viral capsid to allow CAR-independent gene transfer. The first stage of Ad infection involves an interaction between the Ad fiber and CAR. However, this interaction per se is not essential for infection and only serves as a means of bringing the viral particle into intimate contact with the cellular surface. Other cellular receptors could

serve the same role. Therefore, changes to the virion to disrupt CAR binding and to redirect binding to other cellular receptors would allow for a CAR-independent, targeted vector. This has been attempted by two general approaches. One approach involves conjugate-based strategies, which require a multi-component system in which Ad is complexed with targeting molecules. A second approach is genetic targeting, in which the viral particle is genetically modified, thus forming a single-component system.

3.1. Conjugate-based strategies

3.1.1. Bispecific chemical conjugates

One approach to targeting Ad vectors to specific cellular receptors involves complexing bispecific targeting conjugates with the Ad vector (Fig. 3). This has taken on various forms. The first strategy was described by Douglas et al. [50] and involved redirecting Ad infection by employing a bispecific conjugate consisting of the Fab fragment of a neutralizing anti-knob monoclonal antibody (mAb) covalently linked to folate. This proof of principle study showed that a bispecific conjugate complexed to Ad could concurrently ablate native tropism and provide novel tropism, resulting in a truly targeted vector. Another bispecific conjugate consisting of the anti-knob Fab fragment covalently conjugated to the basic fibroblast growth factor (FGF2) has been shown to enhance Ad infection of otherwise poorly infected Kaposi's sarcoma cell lines [51] and vascular endothelial and smooth muscle cells [52]. The fact

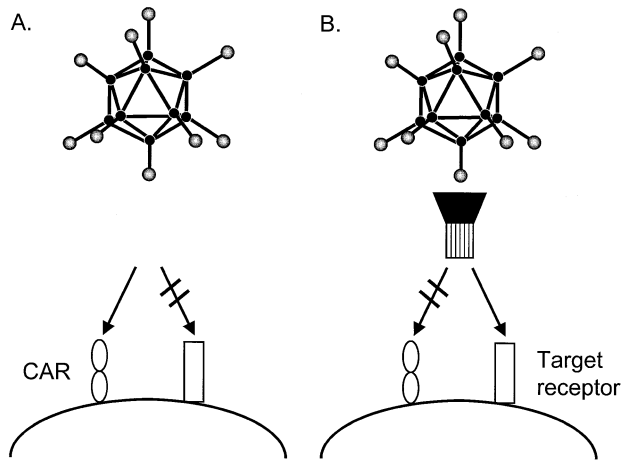


Fig. 3. Retargeting of adenoviral vectors by bispecific conjugates. (A) Ad attachment to cells is accomplished by the high affinity binding of the knob domain of the fiber to the primary cellular receptor, CAR. (B) The bispecific conjugate is designed to simultaneously ablate native tropism, by preventing the Ad vector from binding to CAR, and introduce novel tropism, by directing the vector to a novel target receptor on the cell surface.

that the Fab-FGF2 conjugate has been shown to decrease the dose of Ad needed to mediate infection of a given number of ovarian cancer cells [53,54] has been exploited for therapeutic advantage. Rancourt et al. [54] demonstrated that use of the Fab-FGF2 conjugate to redirect an Ad vector expressing the herpes simplex virus thymidine kinase (HSV-tk) gene improved survival in a murine model of ovarian cancer. Similar results have been reported by other groups, with the additional findings of decreased liver toxicity and hepatic transgene expression [55,56]. A further application of Fab-FGF2-retargeted Ad encoding platelet-derived growth factor-B has been shown in tissue repair [57].

The anti-knob Fab fragment can be used in other forms of conjugates as well. One such approach involves the covalent conjugation of the anti-knob Fab fragment to an antibody fragment directed against a specific cellular receptor. A bispecific conjugate containing an mAb directed against the epidermal growth factor receptor (EGFR) has been employed to retarget Ad to primary and established glioma cells [47] and squamous cell carcinoma of the head and neck [58]. This allowed EGFR-specific infection while also increasing gene transfer to CAR-deficient cells by up to 66-fold over untargeted vector. Additionally, this retargeted vector was selective for tumor relative to noncancerous tissues from the same patient [58].

DCs have been targeted by employing the anti-knob Fab fragment conjugated to an anti-CD40 mAb fragment [44]. This showed an increase in DC gene transfer with subsequent maturation of DCs. Importantly, CD40-based Ad vector targeting to DCs enhanced the efficiency of DC-based vaccination against human papillomavirus 16-induced tumor cells in a murine model [59].

Other cell types which have been targeted by Fab-mAb conjugates include primary ovarian cells via an anti-TAG-72

mAb conjugate, with subsequent 200-fold increase in specificity of gene transfer to tumor relative to untargeted vector [60], and epithelial carcinoma cells via an anti-epithelial cell adhesion molecule (EpCAM) mAb conjugate [61,62]. B cells have been targeted via an anti-CD-70 mAb conjugated to a complete anti-fiber mAb with a resultant 10- to 20-fold increase in infection of Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines relative to untargeted vector [63].

Reynolds et al. [64] targeted pulmonary endothelial cells *in vivo* via a systemically administered anti-angiotensin converting enzyme (ACE) mAb conjugate. This established that an Ad vector complexed with a bispecific antibody maintains its targeting ability *in vivo* and thus provides for the exciting possibility of systemic delivery of a multi-component Ad vector.

The versatility of the anti-knob Fab has been shown by chemical conjugation to the H_c fragment of tetanus toxin, permitting neuronal cell targeting [65]. Furthermore, the anti-knob Fab has been conjugated to a synthetic lung-homing peptide identified by phage display [66].

In each case, the vector was able to ablate the native tropism of Ad and introduce novel tropism by binding to a cellular receptor other than CAR. This further illustrates that CAR is merely a “docking protein” and not essential for Ad infection.

Bispecific conjugates have also been directed against other capsid proteins. Wickham et al. [67] incorporated the FLAG peptide into the Ad penton base and employed full-length anti-FLAG mAb antibodies conjugated to full-length anti- α_v integrins to target endothelial and smooth muscle cells with a 7- to 9-fold augmentation of gene transfer. Importantly, the use of full-length mAbs allows for extension of the targeting moiety beyond the fiber knob, thereby rendering it accessible to cellular receptors. This strategy was also employed to target Ad-resistant T cells via an anti-CD3 mAb, which resulted in a 100- to 500-fold increase in gene transfer [68], and endothelial cells via an anti-E-selectin mAb, yielding a 20-fold increase in gene transfer [69]. Again, these studies demonstrate that Ad vectors do not need to interact with CAR, but rather only need to dock to a cellular receptor in order to infect α_v -positive cells. Yoon et al. [70] derived a bispecific conjugate by employing the Fab fragment from an anti-hexon mAb and conjugating it with an anti-AF-20 mAb specific for the AF-20 antigen found on the FOCUS hepatocarcinoma cell line. While this approach does expand tropism, it does not ablate CAR binding and is therefore not truly targeted.

While these chemically linked bispecific conjugates offer the ability to readily target alternative cellular receptors for proof of principle type studies, their synthesis yields a population of conjugates which is heterogeneous, thereby complicating their use in clinical trials. Moreover, the efficiency of chemical conjugation can be low. To address these problems, targeting studies have been conducted with

bispecific targeting moieties that yield a homogeneous population, and therefore meet regulatory requirements.

3.1.2. Bispecific recombinant fusion proteins

Recombinant bispecific fusion proteins are based on the same design principle as chemical conjugates. As such, one component of the protein is directed against the Ad capsid, while the second component is directed against the cell surface protein. In one approach, a neutralizing anti-Ad knob single-chain antibody (scFv) is genetically fused to a cell receptor-specific ligand or scFv. This offers the ability to neutralize the fiber knob, while allowing binding to the target receptor. Watkins et al. [71] were the first to describe what they called an “adenobody” which contained an anti-knob neutralizing scFv with the epidermal growth factor (EGF) fused to its C-terminal. This fusion protein was able to bind both the fiber knob and EGFR, resulting in a truly targeted vector by ablating CAR binding and redirecting to EGFR. Nicklin et al. [72] expanded on this concept by fusing the anti-knob scFv to a peptide, isolated by phage display, capable of selective binding to human umbilical vein endothelial cells (HUVECs), resulting in a 15-fold increase in transduction of these CAR-deficient cells relative to untargeted Ad. Another approach was demonstrated by Haisma et al. [73] in which an anti-knob scFv was fused with an anti-EGFR scFv. This bispecific scFv was able to redirect Ad infection to a panel of CAR-deficient, EGFR-expressing cell lines [73] and also showed an up to 11-fold enhancement of gene transfer to primary glioma cells and spheroids [74]. Another bispecific scFv has been produced by Nettelbeck et al. [75] in which the anti-knob scFv was fused with an anti-human endoglin scFv fragment, which bound specifically to proliferating primary endothelial cells or cell lines. One disadvantage of employing scFvs in targeting conjugates is the inability to reliably predict if a given scFv will yield a soluble conjugate which can be purified and retain function.

Another class of bispecific fusion proteins utilizes the extracellular domain of CAR as the knob-binding moiety instead of the anti-knob scFv. This also provides for a truly targeted vector because an Ad vector will be unable to bind to CAR expressed on the cellular surface once it is complexed with a CAR–ligand fusion protein. Dmitriev et al. [76] genetically fused EGF at the C-terminal of the extracellular domain of CAR, which resulted in a soluble, bispecific fusion protein. This CAR–EGF fusion protein was able to redirect Ad infection to a panel of EGFR-expressing ovarian, squamous cell carcinoma, and epidermoid carcinoma cell lines with an 8-, 10- and 50-fold increase in gene transfer, respectively [76]. This fusion protein was later shown to mediate Ad infection in an EGFR-specific, CAR-independent fashion to primary and established pancreatic carcinoma cell lines with up to a 5-fold increase in gene transfer relative to untargeted vector [77]. Another derivation of soluble CAR-based fusion proteins was shown by Ebbinghaus et al. [78] in which the extracellular domain of CAR was fused with the

Fc region of the human immunoglobulin G1 protein. This allowed a 250-fold increase in targeted infection of CAR-negative human monocytic cell lines expressing the high-affinity Fc γ receptor I (CD64).

3.1.3. Bispecific peptides

Other conjugate-based strategies have been explored, but to a lesser degree. Hong et al. [79] have retargeted Ad infection by employing a bispecific peptide. This 35-mer peptide consisted of one 20-mer peptide region, which recognized the receptor-binding region of Ad5 knob, and another peptide region which corresponded to the gastrin-releasing peptide. Unlike other fusion proteins, this bispecific peptide was not complexed to the Ad prior to infection, but rather was bound to the cellular receptors prior to infection *in vitro* [79]. Therefore, the feasibility of this strategy *in vivo* is uncertain.

3.1.4. Polymer-mediated ligand coupling to Ad capsid

Another scheme to link targeting ligands to Ad involves the use of polyethylene glycol (PEG). This strategy has important implications for Ad vectors above and beyond targeting, since PEGylation of Ad vectors provides protection from neutralizing antibodies. Romanczuk et al. [80] used PEG to couple Ad to a peptide which targets ciliated epithelial cells and Drapkin et al. [81] used PEG to couple Ad to a urokinase plasminogen activator-derived peptide. In both of these cases, the tropism of the vector was expanded, but native tropism was not ablated. Fisher et al. [82] described a covalent coating and retargeting strategy employing a multivalent hydrophilic polymer with FGF2 and vascular endothelial growth factor (VEGF) incorporated as targeting ligands. This vector exhibited ligand-mediated, CAR-independent gene transfer as well as resistance to Ad neutralizing antibodies.

3.1.5. Biotinylated Ad–avidin bridge

A three-component strategy has been demonstrated by Smith et al. [83] in which an avidin bridge was used to link biotinylated Ad to biotinylated stem cell factor (SCF), a ligand for the c-Kit receptor that is expressed on primitive human hematopoietic cells. This produced a 2440-fold increase in gene transfer as compared to the control vector consisting of a biotinylated Ad linked to avidin alone. Substitution of the biotinylated SCF with biotinylated antibodies directed against c-Kit, CD34, or CD44 increased gene transfer 50-, 8-, and 260-fold, respectively, as compared to the control. Likewise, Kreda et al. [84] employed a streptavidin bridge to link a biotinylated Ad to biotinylated UTP, an agonist for the P2Y₂ receptor expressed on well-differentiated airway epithelium. This vector was able to infect the Ad-resistant airway epithelia cells in a targeted manner. The disadvantage of this system is that it involves three components, thus production of such a vector system on a large scale would involve adhering to regulatory requirements which would be much more complex.

Overall, the advantages offered by conjugate-based strategies include the wide range of cellular receptors which can be targeted by scFv- or ligand-based targeting moieties, along with the fact that there is no need to make structural changes to the Ad itself. Hence, a single targeting molecule can be used with many different vectors. However, since conjugate-based strategies involve two components that must be produced independently and then complexed together, there is a possibility of batch-to-batch variation between lots of conjugate-based targeted vectors. Also, each individual component plus the complexed vector must meet regulatory approval prior to clinical use. However, a strategy which involves only one component would not have this problem. Such a single-component vector is possible by genetic modification of the Ad capsid.

3.2. Genetic targeting strategies

Targeted Ad vectors have been generated by modifications of the fiber, penton base and hexon capsid proteins. Since the knob domain of the fiber is responsible for binding of Ad to the native primary receptor, most attempts to retarget Ad have focused on modifications of the fiber.

3.2.1. Genetic fiber modifications

3.2.1.1. Fiber- and knob-pseudotyping. While the most commonly used Ad vectors for gene therapy are based on subgroup C serotypes 2 and 5, which recognize CAR, other Ad serotypes recognize a different primary cellular receptor. This has led to the hypothesis that CAR-independent gene transfer could be accomplished by substituting fiber genes from the Ad2 or Ad5 backbone with genes encoding fiber proteins from alternate Ad serotypes, a process known as pseudotyping (Fig. 4). This approach is facilitated by the

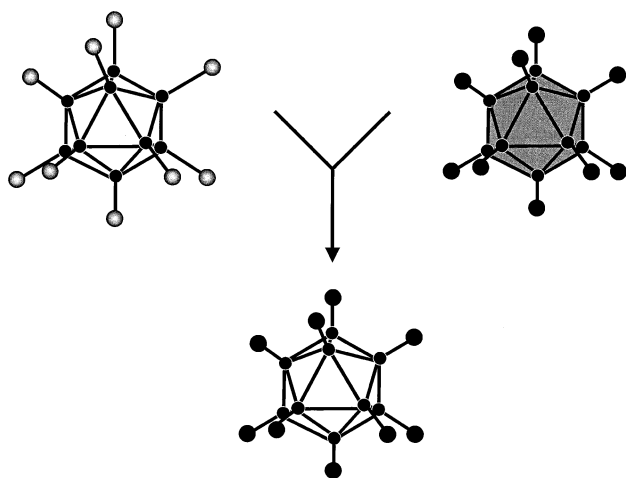


Fig. 4. Schematic diagram of fiber-pseudotyped vector. The fiber-pseudotyped vector is constructed by substituting the fibers from an Ad2 or Ad5 vector backbone with the fiber proteins from an alternative serotype.

structural similarity between fibers of different Ad serotypes, highlighted by the high degree of homology between the fiber tail domains. This suggests that the fiber tail of one Ad serotype could be incorporated efficiently into the capsid by association with the penton base of a different Ad serotype.

This was first demonstrated by Gall et al. [85] by replacement of the native fiber gene in an Ad5 vector with the fiber gene of Ad7. The removal of the Ad5 fiber and subsequent substitution with the Ad7 fiber resulted in altered viral tropism.

The application of this technology was demonstrated by two groups that conducted screening studies for Ad serotypes which showed an enhanced ability to infect a given cell type. The ability of Ad17 to infect well-differentiated ciliated human airway epithelia (CHAE) or fetal rat central nervous system (CNS) cells more efficiently than Ad2 was transferred to an Ad2-based vector by pseudotyping with the Ad17 fiber. This vector demonstrated a 15- to 95-fold increase in gene transfer to CHAE cells [86] and a 7-fold increase to CNS cells as compared to the unmodified Ad2 in vitro [87].

Shayakhmetov et al. [43] targeted human hematopoietic stem cells (HSCs) by analyzing the ability of a panel of Ad serotypes to infect CD34+ cells, with subgroup B Ad35 generating the best results. An Ad5-based vector incorporating the Ad35 fiber showed a 7-fold increase in transduction of CD34+ cells relative to the unmodified Ad5, as well as enhanced infection of CD34+, CD34-, CD38- and Hoechst negative 'side population' marrow cells [88]. Also, an Ad5 vector incorporating a subgroup B Ad11 fiber was used to infect a model for human hematopoietic progenitor cells [89].

Other investigators have substituted the Ad5 fiber shaft and knob domains with the corresponding domains from other fibers of other serotypes. Rea et al. [90] found that Ad35 was efficient at mediating infection of human DCs, and the resulting vector consisting of an Ad5 backbone with the Ad35 fiber shaft and knob showed an enhancement of gene transfer 100-fold higher than the Ad5 alone. However, this infection did not result in maturation of DCs as did a CD40-targeted vector [44]. Subgroup B Ad16 was found to be more efficient at infection of cardiovascular and synovial tissues than Ad5. An Ad5-based vector containing the Ad16 fiber shaft and knob domains yielded an 8- and 64-fold increase in gene transfer to endothelial and smooth muscle cells as compared to Ad5 [91] and an 150-fold increase in gene transfer to cultured synoviocytes as compared to Ad5 [92].

Replacement of only the knob domain of the fiber can also alter viral tropism. This has been demonstrated by replacement of the Ad5 knob with the subgroup B Ad3 knob [93]. This resulted in an Ad5/3 chimeric fiber which conferred altered tropism on the vector. Stevenson et al. [94] demonstrated that Ad5/3 pseudotyping improved gene delivery to human fibroblasts and head and neck cancer

cells when compared to unmodified Ad5. Likewise, Von Seggern et al. [95] derived a vector with an Ad5/3 chimeric fiber that showed at least 10-fold increase in infectivity of EBV-transformed B lymphocytes as compared to Ad5.

The simplicity with which an Ad fiber can be genetically modified with only minimal perturbations to the fiber structure is a major advantage of pseudotyping. Also, since patients are less likely to have been exposed to Ad serotypes other than the commonly occurring Ad5, the likelihood of preexisting neutralizing anti-fiber antibodies in the serum is low. However, the disadvantage of a vector containing a pseudotyped fiber is similar to the disadvantage of a vector with a native fiber. The tissue distribution of the receptor for the fiber limits the targeting potential of a pseudotyped vector, resulting in a vector that is not truly targeted. However, one approach to overcome this limitation is by incorporating cell-specific targeting ligands into the fiber to redirect Ad infection.

3.2.1.2. Genetic incorporation of targeting ligands into the fiber protein. The structure of the fiber suggests that the CAR-binding knob domain is a logical location for the incorporation of ligands to target Ad infection. However, some basic guidelines should be followed in order for the modifications to be successful. First, incorporation of ligands should not disrupt the ability of the fiber to trimerize. Also, the targeting ligand needs to be expressed on the surface of the fiber to allow access to its cognate receptor.

Initial modifications to the Ad5 fiber protein were performed prior to elucidation of the crystal structure. However, the N-terminal tail was known to be associated with the penton base while the C-terminal projected from the surface of the virion [8]. Therefore, modifications were made to the C-terminal of the fiber tail. Michael et al. [96] were the first to show that a peptide ligand, the gastrin-releasing peptide (GRP), could be genetically fused by a flexible linker to the C-terminal of the fiber protein without disruption of fiber trimerization. A recombinant fiber protein incorporating the genetically fused GRP was able to maintain its interaction with a GRP-specific antibody.

Wickham et al. [35] subsequently demonstrated the incorporation of a heparan-binding domain consisting of a stretch of lysine residues at the C-terminal. This vector was able to efficiently infect a panel of Ad5-refractory cell lines, resulting in a 9- to 311-fold increase in gene transfer. This group later developed a refinement of this vector by making a vector containing seven lysine residues, named AdZ.F(pK7), and also constructed a vector named AdZ.F(RGD) incorporating an arginine–glycine–aspartic acid (RGD) peptide at the C-terminal to target α_v integrins, [36]. AdZ.F(RGD) increased gene delivery to α_v integrin-positive endothelial and smooth muscle cells. AdZ.F(pK7) increased infection 5- to 500-fold in a variety of CAR-deficient cell types including macrophages, endothelial, smooth muscle, fibroblast, and T cells. The authors investigated the ability of the AdZ.F(pK7) vector to infect vascular smooth muscle in a pig model in which the

left and right iliac arteries were damaged by a balloon catheter and then transduced for 30 min with no blood flow. Blood flow was then reestablished. The AdZ.F(pK7) vector did show an increase in gene transfer as compared to an unmodified vector, although the increased efficacy was less than would have been predicted from the in vitro experiments [36].

Subsequent studies of the infection efficiency of specific cell types by these vectors have been performed. Notably, AdZ.F(RGD) was more efficient at in vivo gene transfer to the renal cortical vasculature in rats than an unmodified Ad [97], while AdZ.F(pK7) showed enhanced gene transfer to skeletal muscle [98], myeloma cells [99], primary acute myelogenous leukemia blast cells [100], and glioma cell lines [101] when compared with unmodified Ad.

Another vector composed of 20 lysine residues fused via a linker peptide to the C-terminal of the fiber was first described by Yoshida et al. [102]. This vector backbone has been employed to deliver various therapeutic genes in a strategy to target malignant glioma [102,103], resulting in an increase in gene transfer as compared to unmodified vectors.

Although modifications of the C-terminal have been demonstrated as a strategy for targeting Ad vectors, some attempts at incorporating ligands have been unsuccessful [36]. It has been shown that a 27-amino-acid addition to the C-terminal of the fiber results in an inhibition of fiber trimerization [14]. Consequently, this presents a limitation to the length and therefore the variety of targeting ligands capable of being employed in targeting strategies involving C-terminal modifications. Thus, there is a need to find alternative methods of genetic fiber modification.

The resolution of the crystal structure of the Ad5 knob allowed Xia et al. [104] to propose a model in which each monomer of the trimeric knob consists of a series of β -strands connected by flexible loops. This led Krasnykh et al. [105] to hypothesize that the so-called HI loop, which connects β -strands H and I, is a logical location for the insertion of targeting ligands. The HI loop is localized on the surface of the knob, possesses a degree of flexibility, suggesting that an incorporated targeting ligand could fold into its correct configuration, and is not involved with intramolecular interactions which may affect the stability of the fiber. In addition, the length of this loop in different Ad serotypes is variable, potentially allowing a wide variety of targeting ligands to be exploited.

This logic led Krasnykh et al. [105] to incorporate a ligand, the octapeptide FLAG, into the HI loop in order to test the potential of this site for the genetic modification of the Ad fiber knob. This proof of concept study revealed that a genetically modified fiber protein incorporating the FLAG peptide retained its native trimeric configuration. The FLAG peptide was able to bind to an anti-FLAG mAb upon incorporation of the chimeric fiber-FLAG protein into an Ad vector. This vector retained the ability to bind and infect CAR-positive cells via a CAR-mediated pathway, indicating that native tropism had not been abolished [105]. Taken

together, these findings provided the rationale for development of a vector containing a targeting ligand in the HI loop.

Dmitriev et al. [45] then derived a vector incorporating the targeting peptide RGD-4C (CDCRGDCFC) into the HI loop of an Ad5 vector. This ligand was chosen for its small size and ability to bind with a number of cellular integrins [106,107]. This vector possessed expanded tropism since it retained the ability to recognize CAR, but was able to infect cells via a CAR-independent, α_v integrin-mediated pathway, resulting in 2 orders of magnitude of increased gene transfer to CAR-deficient primary ovarian cancer cells [45].

Ad vectors containing the RGD motif in the HI loop have subsequently been shown to increase the efficiency of gene delivery by up to 3 orders of magnitude to a variety of CAR-deficient cancer cells including primary [108] and established ovarian cancer cells [108,109], squamous cell carcinoma of the head and neck [110], myelomonocytic leukemia [111], rhabdomyosarcoma [112], glioma [74,113] and pancreatic cancer cells [77], as well as DCs [114], primary vascular smooth muscle cells [109], and primary human endothelial cells [109]. These findings demonstrate the ability of the RGD-modified vector to increase gene transfer to cells that would otherwise be Ad-refractory due to a paucity of CAR. Importantly, this vector has also been found to circumvent inhibition by neutralizing anti-fiber antibodies which block infection by unmodified Ad vectors [115,116].

Mizuguchi et al. derived a vector containing the asparagine–glycine–arginine (NGR) motif in the HI loop. This peptide is recognized by aminopeptidase N (CD13) [117]. The vector containing the NGR peptide showed a 100-fold increase in gene transfer to CAR-deficient glioma cells as compared to unmodified vector [113].

Xia et al. [118] employed phage panning to identify 10 candidate peptides to target the human transferrin receptor (hTfR), a molecule found at high density on human brain microcapillary endothelium. Incorporation of these motifs into the HI loop did not inhibit trimerization of the fiber. However, the incorporation of these peptide sequences was not well tolerated in all of the vectors. Three of the vectors were unable to be propagated and required the use of cell lines expressing a surrogate receptor and two of the seven vectors that did allow for propagation were very difficult to produce. This indicates that incorporated peptides may disrupt the fiber structure, thereby abrogating the CAR-binding capacity of the vector. Nonetheless, the fiber-modified viruses did increase gene transfer by 2- to 34-fold in hTfR-expressing cell lines [118]. Another potential problem with incorporation of targeting peptides into the fiber knob is that peptides can change their three-dimensional architecture upon inclusion in the viral protein, thereby altering their receptor-binding specificity [119].

To date, the insertion of targeting ligands into the knob domain has led to the expansion of viral tropism, but not necessarily the ablation of CAR binding. The resolution of the crystal structure of the Ad fiber knob domain in complex

with CAR [18] along with mutagenesis studies [120,121] have identified key determinants of binding specificity. Mutagenesis of the amino acid residues involved in CAR recognition should allow the development of truly targeted Ad vectors.

3.2.1.3. Genetic fiber replacement strategies. The recognition of the limitations imposed on the design of targeted vectors by the structural constraints of the fiber knob has led to the more radical approach of Ad targeting by replacement of the fiber or knob. Strategies to replace the fiber protein must incorporate the essential functions of the knob domain, namely receptor binding and trimerization. The purpose of replacing the C-terminal knob domain is to ablate CAR binding and introduce novel tropism by incorporating ligands in such a way that targeting motifs of sufficient size and variety can be employed without causing vector dysfunction. However, the molecule chosen to replace the fiber must be able to self-assemble into a trimer, since the knob domain initiates trimerization in the native fiber. Likewise, the association with the penton base makes the inclusion of the trimeric N-terminal of the native fiber vital to any genetic replacement approach. Additionally, chimeric fibers must be able to localize to the nucleus for incorporation into the viral capsid.

Krasnykh et al. [122] generated an Ad vector lacking wild-type fibers but instead containing a fiber chimera consisting of the tail domain and two pseudorepeats of the N-terminal of the Ad5 fiber genetically fused to the trimeric bacteriophage T4 fibrin protein, with its N-terminal deleted. Previous reports have described the use of T4 fibrin for ligand display [123,124], suggesting that a wide variety of targeting ligands could potentially be exploited by this approach. In this study, a six-histidine (6His) tag was connected to the C-terminal of the fibrin protein via a short peptide linker. The 6His tag functioned as a targeting motif. This chimeric protein was trimeric and could be incorporated into a stable virion capable of CAR-independent infection of target cells expressing an artificial receptor, an scFv specific for 6His. Although fiber replacement did not affect the stability of the virus or its yield, the efficiency of infection by the virus with chimeric fibers was lower than that of the control virus with wild type fibers [122]. Krasnykh et al. suggested that the reduced efficiency of infection might have been due to the lower affinity of the 6His tag for its target receptor. However, studies with fiberless Ad particles have indicated that the fiber protein may function in the maturation of the virus particle. In this regard, Legrand et al. [125] have suggested that the biological activity of the 23 K protease, which is responsible for processing viral precursors and is required for virus entry into cells, is altered in the absence of the fiber. This results in the production of immature virions that are then impaired in later steps of the cell entry pathway, leading to a decrease in gene transfer.

An attempt was reported by van Beusechem et al. [126] to replace the fiber knob with the α -helical coiled coil trimerization domain from the Moloney murine leukemia virus (MoMuLV) p15 envelope glycoprotein. These fiber chimeras consisted of the entire Ad5 fiber tail and shaft domains and a trimerization domain derived from the MoMuLV envelope glycoprotein linked to the targeting motifs Myc-epitope and 6His. The ensuing fiber chimeras were relatively unstable, which was not unexpected due to the low thermostability of p15. However, these fibers did localize to the nucleus for capsid assembly and were incorporated into mature Ad virions, along with wild-type fibers [126].

Magnusson et al. [127] constructed Ad vectors containing fiber chimeric proteins which replaced the knob and 21, 15, or 0 fiber shaft repeats with the neck region peptide (NRP) of human surfactant protein D as an external trimerization motif fused to the N-terminal tail of the Ad5 fiber. The RGD motif was employed as the targeting ligand by linkage to the C-terminal of the NRP via a sequence from *Staphylococcus aureus* protein A. These fibers were named R1-RGD, R7-RGD, and R22-RGD, respectively. Studies of the chimeric fibers revealed that the R22-RGD fiber was expressed at low levels and the R1-RGD self-assembled into unstable fiber trimers. The vector incorporating the R7-RGD fiber, Ad5/FibR7-RGD, was found to yield plaques on 293 cells, but less efficiently than wild type Ad5. Moreover, the ratio of infectious particles to physical particles for the chimeric fiber vector was 20-fold lower than wild type Ad5. Tropism of the Ad5/FibR7-RGD was expanded to CAR-negative, integrin-positive cells, but the infectivity index revealed that Ad5/FibR7-RGD was 20 times less infectious than wild-type vector [127].

These proof of principle reports demonstrate the feasibility of fiber- or knob-replacement strategies for Ad targeting. This offers the advantage of knob removal, resulting in ablation of native tropism, so that the introduction of a targeting ligand will produce a truly targeted vector. Also, the provision of trimerization by distinct domains in a chimeric protein has the potential to allow the incorporation of large targeting moieties, perhaps even an scFv. However, knob replacement may also decrease the stability of the vector itself or alter the efficiency of gene transfer.

3.2.2. Propagation of truly targeted Ad vectors

While the development of truly targeted vectors will greatly improve upon their clinical utility, CAR-binding ablated vectors present a unique problem. The inability of these vectors to bind CAR indicates that they cannot be amplified in standard packaging cell lines in which propagation is dependent on the native cellular entry pathway. Therefore, alternative approaches have been developed to allow for high titer vector production.

To overcome the difficulties of propagating CAR-ablated vectors, two groups have developed a strategy employing a

vector containing a peptide motif with no known natural cellular receptor and cells expressing an artificial receptor specific for this peptide. Douglas et al. [128] described the incorporation of 6His residues at the C-terminal of the Ad fiber while the extracellular domain of the artificial receptor consisted of an anti-His scFv. A similar system has been demonstrated that employs a hemagglutinin (HA) epitope incorporated into the Ad capsid with an anti-HA scFv expressed on cells functioning as the complementary receptor [129]. While this system does have the disadvantage of requiring the vector to be designed to include the propagation motif, this can be overcome by incorporating the peptide into a prototype vector. This would then allow for subsequent modifications to introduce new targeting ligands and ablate native tropism.

Another approach to develop a production system for CAR-binding ablated vectors involves the use of CAR-positive cell lines modified to stably express the wild-type Ad5 fiber protein. This would allow for the production of fiber-mutated vectors in CAR-expressing cell lines by incorporation of the wild-type trimeric fiber into the viral capsid during the early rounds of infection for vector propagation [125,130]. The final round of propagation employs cells that do not express the wild-type fiber, resulting in vectors which possess only the fiber mutations. One potential disadvantage of this approach is the theoretical possibility of recombination with resultant restoration of the wild type fiber gene in the viral genome, resulting in loss of targeting specificity.

3.2.3. Genetic modifications of other capsid proteins

Attempts to target Ad vectors have also included genetic modifications of the penton base or hexon capsid proteins, although these proteins have not been exploited to the same extent as fiber-based targeting strategies. This is mainly because these proteins do not play a significant role in attachment of Ad to primary cellular receptors, so their utility for retargeting is less obvious.

Wickham et al. [131] generated penton base chimeric proteins with mutations of wild-type RGD that altered the ability of $\alpha_v\beta_3$ - and $\alpha_v\beta_5$ -positive cells to adhere to the penton base protein. Replacement of the RGD with the $\alpha_4\beta_1$ integrin-binding motif, LDV, led to an ability to redirect adherence away from $\alpha_v\beta_3$ -expressing epithelial cells and to $\alpha_4\beta_1$ -expressing T- and B-cells. These modifications resulted in alterations to the binding specificity of the penton base protein and suggests that penton base modifications can be employed as a means to redirect Ad binding [131].

Fiber-independent cellular attachment of Ad via binding of the penton base to cellular integrins has been demonstrated. This was shown with Ad9, which has short 11-nm fibers as compared to the longer 37-nm fibers of Ad2 and Ad5. This suggests that the shortening of Ad fibers may be a useful strategy to mediate penton base-directed Ad infection [132]. However, Huang et al. [40] demonstrated that Ad2

with its 37-nm fibers binds to β_2 integrins via its penton base. This suggests that a short fiber is not necessary for penton base-facilitated binding. This was further supported by a study with incorporation of the HA epitope into the RGD-containing loop of the penton base and subsequent binding to CAR-negative cells expressing an artificial receptor recognizing HA [129].

Although the hexon does not play a documented role in Ad5 cell entry, the hexon protein is an attractive candidate for genetic targeting modifications because of its abundance in the Ad capsid. Additionally, hypervariable regions [133] that are localized on the surface of the Ad virion [134] represent rational sites for the incorporation of targeting ligands. The feasibility of this strategy was demonstrated by Vigne et al. [135] by replacement of one of the hypervariable regions with a peptide containing the RGD motif flanked by flexible linkers. This vector was able to infect vascular smooth muscle in a fiber-independent fashion.

Two of the minor structural components of the Ad capsid, polypeptide IIIa (pIIIa) and IX (pIX), have also been studied for incorporation of targeting ligands. Dmitriev et al. [136] modified Ad vectors so that pIIIa expressed an N-terminal 6His tag and pIX expressed a C-terminal FLAG octapeptide. Both were incorporated into mature virions and did not disturb viral assembly. The N-terminal addition of 6His to pIIIa was not accessible for binding, but the C-terminal addition of FLAG to pIX was accessible for binding in the context of the mature viral particle. Incorporation of an RGD peptide in the C-terminal of pIX resulted in increased gene transfer to CAR-negative cells. This suggests that pIX has the potential to be employed in capsid modification strategies for Ad targeting, although the size of ligands which can be accommodated at this site remains to be determined.

4. Ad vectors as therapeutic agents for clinical gene therapy

The analysis of the utility of any potential therapeutic agent must include an evaluation of that agent's therapeutic index, the ratio of the dose that is likely to produce toxic effects compared with the dose required to produce therapeutic effects. Current Ad vectors have a relatively poor therapeutic index. As discussed above, the poor efficiency of Ad vectors in several clinical trials [137] has been correlated with a low level of CAR expression on the target tissues [45–49]. Hence, targeted Ad vectors capable of efficient and cell-specific CAR-independent gene transfer will be required for clinical gene therapy applications.

It is apparent that additional requirements will be imposed upon targeted Ad vectors designed for clinical use in disease settings for which systemic vector administration is mandated. It has been reported that an intravenously (iv) administered, untargeted Ad5 vector delivers

greater than 90% of the viral dose to the liver [138–140]. This decreases the amount of therapeutic agent available for infection of target tissues. Furthermore, iv administered Ad vectors have been associated with liver toxicity [141,142], and vectors administered by catheter to the liver resulted in the first known death of a participant in a gene therapy trial [143]. Therefore, sequestration of Ad by the liver has a profound negative effect on the therapeutic index of Ad vectors. Hence, a substantial amount of study has been conducted to try to understand the mechanisms involved in vector uptake by the liver.

Zinn et al. [144] demonstrated that uptake of iv administered technetium (Tc)-99m-labeled Ad5 knob by the liver is significantly reduced by coinjection of unlabeled Ad5 knob. Conversely, liver uptake of the Tc-labeled Ad5 knob is not affected by Ad3 knob, which recognizes a different primary receptor. This suggests that uptake of Ad5 by the liver is receptor-mediated. This was supported by the subsequent findings of high levels of CAR mRNA in the liver [16,17]. These findings would seem to indicate that avoidance of liver sequestration of Ad5 requires modifications to prevent CAR-mediated binding. In accordance with this concept, Printz et al. and Reynolds et al. [56,64] observed significantly reduced transgene expression in the livers of mice which were administered Ad5 vectors iv that were retargeted to receptors other than CAR via bispecific conjugates. However, the decreased transgene expression by the liver may not be due solely to retargeting. Complexing bispecific conjugates to Ad vectors may create a particle whose size hinders entry into the small fenestrations of the mouse liver sinusoidal epithelium. Interestingly, the biodistribution of Ad DNA 90 min post iv injection was not significantly changed as compared to untargeted virus [64].

Additional studies have also suggested that avoidance of CAR binding in the liver is not the only mechanism by which to avoid hepatic transgene expression. Leissner et al. [145] observed that genetically modified CAR-binding-ablating vectors administered iv did not significantly reduce hepatic transgene expression when compared to unmodified Ad. It should be noted that while the vectors were CAR-binding ablating, they did not retarget to an alternative receptor. Therefore, hepatic uptake could have been penton base-mediated due to loss of high affinity binding, so the Ad vectors could have infected hepatocytes by default due to lack of a target receptor. Similarly, Alemany and Curiel [146] observed that a vector which had undergone genetic modifications of the fiber knob, rendering it CAR-binding ablating, did not have a different biodistribution but rather an increased persistence in the vascular system when compared to an unmodified vector. Additional studies involving a vector which has been modified so that it is CAR-binding ablating, integrin-binding ablating, and retargeted should prove useful in understanding the mechanisms of Ad infection of hepatocytes.

Whereas the majority of hepatic transgene expression involves hepatocytes, other cells play a significant role in

vector sequestration by the liver. An early innate immune response mediated by hepatic tissue macrophages, named von Kupffer cells (KCs), of the reticuloendothelial system (RES) play an important role in vector elimination. These cells are found in the lining of the liver sinusoids and function to phagocytize particulate matter in the hepatic vasculature. Uptake of Ad vectors by KCs does not lead to transgene expression [147]. It has been shown that 90% of Ad DNA is eliminated by the liver within 24 h of administration and does not lead to transgene expression [148]. Furthermore, inhibition of KCs reduces the elimination of viral DNA with subsequent increases in transgene expression [149,150]. It has been proposed that in mice KCs can efficiently eliminate low doses ($\sim 10^{10}$ viral particles) of Ad vectors, but can become saturated with higher doses (1×10^{11} viral particles) and allow hepatic transgene expression [147]. Also, activated KCs are involved in the release of the proinflammatory cytokine tumor necrosis factor (TNF) [149]. The release of TNF is also important because it activates transcription of interleukin-6 (IL-6) in KCs and hepatocytes [151]. IL-6 is one of the cytokines felt to have contributed to the fatal immune response that resulted in the only death caused by vector-associated toxicity of a patient enrolled in a gene therapy trial [143].

Additional vector designs have recently been explored with an emphasis on improved safety. One such design involves creating a vector that combines a transductional targeting strategy with an approach to restrict therapeutic gene expression to the tissue of interest. This transcriptional targeting approach employs a tissue-specific promoter to drive therapeutic gene expression. Expression of the therapeutic gene may invoke a cytopathic or immunologic response, either intentionally or unintentionally. Therefore, this dual targeting strategy can greatly improve upon the safety of Ad vectors, especially in the context of hepatic toxicity by first directing infection away from CAR-mediated pathways and second, by limiting the amount of therapeutic gene expression in nontarget tissue. This concept has been demonstrated by us in an *in vitro* cancer model [152] and by Reynolds et al. [153] in an *in vivo* mouse model. Importantly, the combination of transductional and transcriptional targeting resulted in a synergistic improvement in selectivity for the target cells. Use of this strategy can improve upon the therapeutic index of Ad vectors by decreasing toxicity from aberrant gene expression while simultaneously improving therapeutic efficacy by retargeting to a cell-specific receptor highly expressed on the tissue of choice.

Decreasing vector-associated toxicity alone must also be accompanied by increased therapeutic efficacy in order to improve the therapeutic index of Ad vectors to optimal levels. One of the obstacles to improved efficacy is physical or anatomical barriers. It has been reported that expression of CAR and α_v integrins does not correlate with Ad vector-mediated gene delivery *in vivo* [154]. Also, the basal lamina acts as a physical barrier to infection of mature skeletal

muscle by untargeted and tropism-expanded Ad vectors [155,156]. This suggests that the anatomical barriers of the endothelium and subendothelial matrix must be overcome in order to achieve organ-specific gene therapy. One strategy to overcome this might be to permeabilize the barriers. In support of this, it has been shown that pretreatment of a rabbit iliac artery with elastase enhances gene transfer to arterial smooth muscle cells after balloon abrasion [157], and administration of proteases prior to intratumoral injection of vectors leads to increased Ad infection [158]. One *in vitro* model has shown that the endothelial monolayer forms a physical barrier to Ad infection of myocytes, which can be partially overcome with α thrombin by increasing endothelial permeability [159]. Cho et al. [160] have demonstrated that increasing the hydrostatic pressure in the vascular compartment by administering Ad vectors in a large solvent volume encourages vector passage out of the intravascular space and enhances infection of mature skeletal muscle. Fortunately, one application of Ad vectors which may not be hindered by anatomical barriers could be in the treatment of solid tumors due to their “leaky” vasculature [161].

Therefore, before Ad vectors can reach their full potential as clinical agents, a favorable therapeutic index must be achieved. In order to accomplish this, strategies to target Ad vectors appear very promising. This is due to the fact that Ad vector targeting has the potential to decrease vector toxicity while increasing therapeutic efficacy, thereby providing substantial improvement to the therapeutic index of Ad vectors. To this end, the University of Alabama at Birmingham is currently employing Ad vectors modified to contain the RGD motif in the HI loop of the fiber knob in the first ever clinical trials utilizing tropism-modified viral vectors. These Phase I clinical trials for ovarian cancer and recurrent cancer of the oral cavity and oropharynx are to evaluate the safety of delivering the HSV-tk and cytosine deaminase genes, respectively, via this vector system. Utilization of these vectors could potentially allow for augmented gene transfer at lower vector doses, thereby leading to increased efficacy and decreased toxicity.

5. Conclusion

The development of gene delivery vectors with refined specificity and improved efficacy is essential if gene therapy is to realize its full potential. Biological features of human Ad offer promise for their use as vectors in gene therapy applications. Unfortunately, another biological feature of Ad, namely the native CAR-mediated pathway of infection, is a hurdle that must be overcome. However, this difficulty may be conquered by the introduction of targeting strategies to render Ad vectors capable of delivering therapeutic genes to tissues of interest, in a manner which does not negate their benefit by causing an unacceptable amount of toxicity. Recent studies have revealed the practicability of employing

targeting to improve upon current Ad vectors. Further gains could be made by combining compatible strategies. An example of this could be the use of two or more vectors targeted against distinct cellular receptors in a heterogeneous population of cells, as might be found in a solid tumor, in order to increase gene transfer and improve therapeutic efficacy [74]. Furthermore, utilization of targeting strategies to redirect replicating adenoviruses is mandated to allow the clinical potential of this class of viral vectors to be reached [162].

The studies described in this review have advanced the science of Ad targeting from a theoretical to an applied branch of experimental virology. However, physiological factors such as anatomical barriers and components of innate immunity that operate to prevent infection from naturally occurring pathogens are barriers to any Ad vector. Future refinement of Ad vectors should yield truly targeted vectors capable of a therapeutically beneficial performance in *in vivo* clinical applications.

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