1. Introduction

Viral vectors show great promise for gene delivery in both basic research and therapeutic applications. It is vital to select the most appropriate viral vector for each specific application, and a number of factors must be taken into consideration when making such a selection. These include the efficiency and specificity with which the vector infects the target cells, the transgene size, the level and duration of the transgene expression, the question of whether regulation of the transgene is needed, and the level of toxicity that can be tolerated. There are now more than 10 viral vector types in use, derived from common human or mammalian viral pathogens including retrovirus, adenovirus, adeno-associated virus, lentivirus, herpes simplex virus, and poxvirus. However, there is no single viral vector type meeting all the requirements, and the methods for using viral vectors to deliver genes are continually being refined.

Adenoviruses (Ad) are nonenveloped viruses containing an icosahedral protein capsid with a diameter of approximately 80 nm. There are at least 51 serotypes of human Ad identified and classified into six different subgroups (A–F), many of which are associated with respiratory, gastrointestinal, or ocular diseases. Of them, Ad serotype 5 (Ad5) along with Ad serotype 2, both belonging to subgroup C, have been the most extensively studied for use as vectors in gene therapy applications. Ad capsids consist 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). 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. Fiber proteins consist of three distinct domains: tail, shaft, and knob. Each domain has distinct functions in host cell infection. The amino-terminal tail anchors the fiber

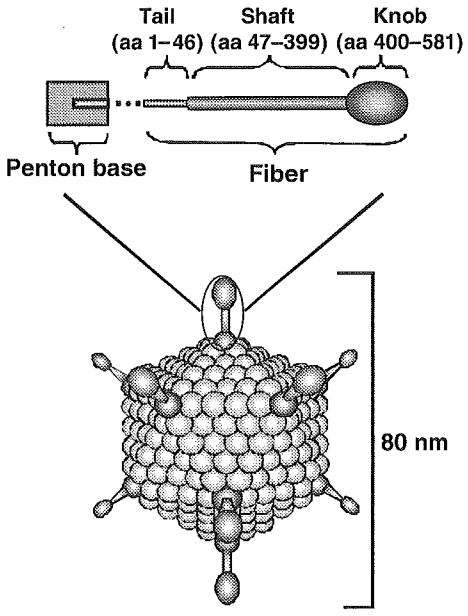

Fig. 1. Schematic diagram of a human Ad serotype 5 virion. The double stranded virus genome is packaged within an icosahedral protein capsid. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices.

to the Ad capsid through association with the penton base [1]. The shaft extends away from the virion surface and, in Ad5, is composed of 22 pseudorepeats of 15 amino acids in a triple-β-spiral conformation [2]. By extending the knob away from the virion, the shaft facilitates its interaction with host receptor [1]. The trimeric subunits of the carboxyl (C)-terminal knob domain are responsible for binding to the host's primary cellular receptor [3,4].

Human Ad5 contains a linear, approximately 36 kb, double-stranded DNA genome encoding over 70 gene products. The viral genome contains five early transcription units (E1A, E1B, E2, E3 and E4), two early delayed (intermediate) transcription units (pIX and IVa2) and five late units (L1–L5), which mostly encode structural proteins for the capsid and internal core. Inverted terminal repeats (ITR) at the end of the viral genome function as replication origins (Fig. 2). The E1A gene is the first transcription unit to be activated shortly after infection, and is essential to the activation of other promoters and the replication of the viral genome. In the first-generation Ad vectors, the E1 (E1A and E1B) gene is deleted and the virus is propagated in E1-transcomplementing cell lines, such as 293 [5], 911 [6], or PER.C6 cells [7]. The E3 region-encoded proteins modulate the host defense, but are not required for viral replication in vitro; thus, the E3 region is often deleted to enlarge the packagable size limit for foreign genes. Since up to 3.2 and 3.1 kb of the E1 and E3 regions, respectively, can be deleted [8], and approximately 105% of the wild-type genome can be packaged into the virus without affecting the viral growth rate and titer [9], E1/E3-deleted Ad vectors allow the packaging of approximately 8.1–8.2 kb of foreign genes [8].

The coxsackievirus and adenovirus receptor (CAR), a broadly distributed type I membrane protein, has been identified as the primary receptor for Ad of subgroups A, C, D, E and F [10–12]. Entry of Ad5 into cells is initiated by the attachment of fiber on the surface of the capsid to the CAR on the cell surface. The affinity of the RGD (Arg-Gly-Asp) peptide at the penton base of the Ad5 capsid to the cell surface molecules of the integrin family, such as $\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_1$, helps mediate the internalization of Ad5 into the cell [13–15]. Furthermore, heparan sulfate glycosamino-glycans have also been reported to serve as primary attachment sites for Ad2 and Ad5 [16]. The abundant expression of these receptors in various cells determines the wide tropism of Ad vectors. Internalized Ad reaches the endosomal pathway and avoids lysosomal degradation. Inside the endosome, a stepwise disassembly program takes place, allowing the Ad to release its genome into the nucleus. During this process, the pH of the endosome decreases, leading to the release of the fiber from the virion and the dissociation of the penton base [17]. The resulting endosome rupture allows viral DNA to escape from inside the degraded capsid and to enter the nucleus. This uncoating process of the Ad starts immediately after internalization and ends 40 min after infection with translocation of the Ad into the nucleus. As early as 60 min after infection, the Ad begins to transcribe its genome in the host cell [18].

Ad vectors are the most efficient class of vector in terms of delivering genes into both dividing and non-dividing cells. They have large packaging ability for foreign genes and can be easily grown to high titers and purified for clinical applications. Furthermore, Ad is nononcogenic, and Ad-related pathology is mostly limited to mild upper respiratory tract infections. All these advantageous features lead to increasing number

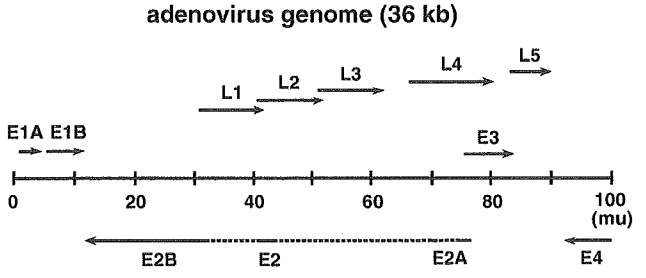

Fig. 2. Genome structure of human Ad serotype 5. The early transcription units E1 (and E3) are deleted for the first-generation Ad vectors.

of clinical protocols employing Ad vectors. As of October of 2003, the percentage of gene therapy protocols utilizing Ad vectors was at 27% (636 protocols) and the percentage of patients treated with Ad vectors at 18% (3496) (Journal of Gene Medicine Website, www.wiley.co.uk/genmed/clinical). This proportion is second only to retroviral vectors.

However, the immune response to the Ad vector-transduced cells dramatically affects the kinetics of Ad vector-delivered genes and the gene products. The potent immunogenic toxicities and consequent short-lived transgene expression of Ad vectors are undesirable properties if Ad vectors are to be more broadly applied. Though Ad vectors find niches in the treatment of degenerative diseases like vascular and coronary artery diseases in which transient transgene expression is advantageous [19,20], and for cancer, in which cellular toxicity and immunogenicity might enhance antitumor effects [21], less antigenic Ad vectors with long-term transgene expression are preferable in most cases. Here, we would like to highlight various approaches to overcoming the hurdle of Ad vector immunogenic toxicities to improve the kinetics of Ad vector-delivered genes and gene products.

2. Immunogenic toxicities of Ad vectors

The greatest obstacle for gene therapists wanting to exploit Ad vectors is the issue of their viral immunogenic toxicities. With the extensive efforts of various research groups, more and more data concerning Ad vector immunogenic toxicities have been obtained. However, it is apparent that the immunogenic toxicities associated with the use of Ad vectors are extremely complex, involving both innate and adaptive immune responses, along with apoptosis, and we are still far from a thorough understanding of all the aspects of the toxic interaction between Ad vectors and their hosts.

The complexity of Ad vector immunogenic toxicities lie in the fact that they induce multiple components of the immune response [22–24]. The cytotoxic T-lymphocyte (CTL) response can be elicited against viral gene products and/or transgene products expressed by transduced cells, resulting in a host immune attack against the transduced cells and the elimination of transgene-expressing cells. The Ad capsid itself can induce humoral virus-neutralizing antibody responses, which prevent transgene expression on subsequent administration of vectors of the same serotype, and which also provoke potent cytokine-mediated inflammatory responses during which NF κB activation might play a central role [25,26]. It is thought that among the inflammatory cytokines, TNF-α plays a dominant role in Ad vector clearance [26,27]. Wilson's group demonstrated that systemically administered Ad vectors preferentially activated dentritic cells and macrophages in the spleen to release inflammatory cytokines, independent of transgene expression [28,29]. This suggests in designing targeted Ad vectors, it should be taken into consideration to reduce transduction of or sequestration by antigen presenting cells.

Like all drug-associated toxicities, the degree to which Ad vectors induce harmful immune-mediated and inflammatory responses and other toxic side effects is dose-dependent [30–32]. Highly dangerous inflammatory responses might be inappropriately activated, especially at high doses of Ad vectors. During the tragic 1999 gene therapy trial for deficiency of ornithine transcarbamylase (OTC) at the University of Pennsylvania (U.S.), an escalated dose of Ad vector (3.8×10^{13} particles) systemically administered through the hepatic artery induced a massive systemic inflammatory response that led to fever, disseminated intravascular coagulation, multi-organ failure and the eventual death of an 18-year-old patient [33,34]. Besides the potential of a lethal outcome, immunogenic toxicities attenuate the therapeutic efficacy by affecting the kinetics of delivered genes and gene products.

3. Kinetics of Ad vector-delivered gene and gene product

It is known that different viruses are cleared from the blood stream by Kupffer cells (KC) [35]. However, blood clearance varies among different viruses [35]. Important determinants of virus clearance from the blood stream include interaction between viral components and cellular receptors, and virion size. The net charge of the viral particle also affects the clearance kinetics [36]. Interventions that slow the

clearance of Ad vectors from the blood stream might favor tissue- or tumor-specific targeting approaches based on systemic delivery [37,38]. Understanding the kinetics of blood clearance and biodistribution of Ad vectors would be beneficial to the advance of their application as therapeutic agents.

Several groups have verified that KC played a central role in clearing the Ad particles from the blood stream by blocking the KC of mouse liver [26,39,40]. The Curiel group's as well as our own, indicated rapid clearance of Ad vectors from the blood of mice, with a half-life of less than 3 min and negligible levels of the Ad vectors remaining in the blood 30 min after injection (Fig. 3A) [38,41,42]. To examine the role of the liver in the blood clearance of Ad vectors, we determined the amounts of Ad vector DNA in the mouse. In accordance with the other groups' data [40,43], we found that 98% of the intravenous dose had accumulated in the mouse liver 1 h after injection. Forty-eight hours after injection, 43% of the input Ad5 DNA persisted in the liver (Fig. 3B) [41]. These data confirm that, following intravenous administration, Ad vectors are predominantly delivered to the liver. Besides the strong interaction between viral components and cellular receptors (e.g. the fiber-CAR and RGD motif of the penton base-αv integrins), the inclination of Ad vectors to the mouse liver may also be attributed to the anatomical properties of the liver sinasoid [43,44]. The accumulation of Ad vectors in the liver may itself be toxic. We further investigated the cellular distribution of Ad vector genomes in mouse livers after intravenous injection, and found them to be equally distributed in the parenchymal cells (PC; hepatocytes) and nonparenchymal cells (NPC; Kupffer cells and endothelial cells), when 1.5×10^{10} particle of Ad vectors were intravenously injected into the mouse [41]. Despite the high uptake of Ad vectors by the NPC, the Ad vector-mediated transduction efficiencies in the NPC were much lower than those in the PC, indicating the uptake of Ad vectors by the NPC is a function of phagacytosis rather than a receptor-mediated infectious pathway [41]. This result of the high uptake of Ad vectors by the NPC combined with low transduction efficiencies is consistent with previous reports of nonlinear dose responses of Ad transduction in the liver [45,46]. Those results suggested that there was a viral dose threshold effect for efficient liver transduction of Ad vectors and that NPC played a central role in this threshold effect, such that low doses of Ad vectors were efficiently taken up by the NPC without appreciable transgene expression, while high doses saturated the NPC and were able to productively transduce the PC. Hence, depleting the Kupffer cells or blocking their uptake before Ad vector administration might be helpful in reducing the Ad vector dose for systemic route gene therapy [45,46].

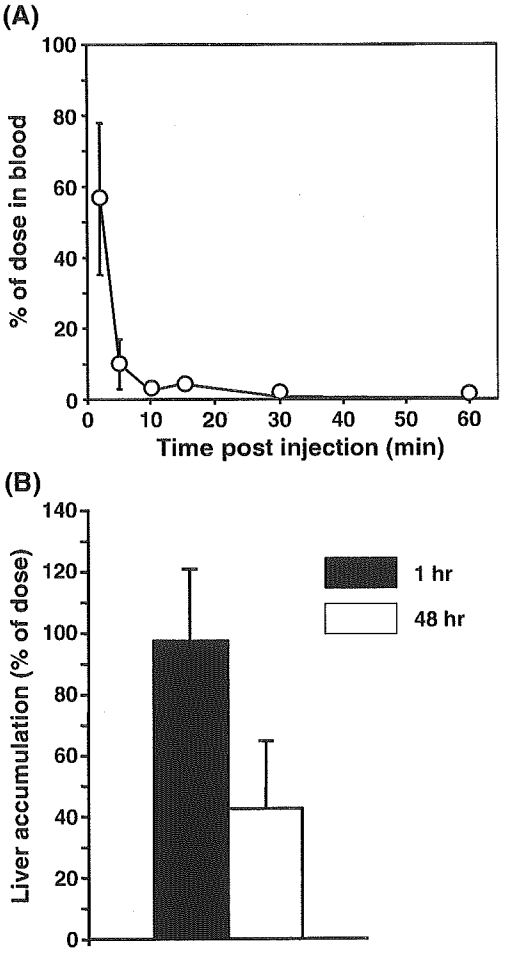

Fig. 3. Blood clearance kinetics and liver accumulation of Ad vectors after intravenous administration into mice. E1- and E3-deleted Ad vectors (1.5×10^{10} particle) were intravenously injected: (A) blood was drawn from the retro-orbital at the indicated times postinjection. (B) The livers were isolated 1 or 48 h after injection. Total DNA, including the Ad vector genome, was isolated from the blood or the livers, and slot-blot analysis was then performed. The data in this figure were published in our previous paper [41].

It is putative that transgene expression from Ad vectors is transient because of the rapid clearance of viral particles by the host immune response. However, duration of transgene expression may vary according to transgene products or animal species/strains. For example, marked variability was observed in the persistence of human alpha 1-antitrypsin (hAAT) expression delivered by an E1/E3-deleted Ad vector in different mouse strains, ranging from several weeks in the strains of C3H/HeJ and Balb/c to more than 3 months in the strains of C57Bl/6, B10.A(2R) and B10.BR [47]. This is because immunogenicity varies according to different transgene proteins, and immune responses (cellular and/or humoral) to invading virions vary according to different species or strains [48].

Overall, the kinetic features of Ad vector-delivered genes and gene products might be summarized as rapid clearance of virus from blood, liver accumulation of virus DNA and expression, and transient transgene expression. Moreover, unlike common-sense pharmaceuticals, readministration of Ad vectors is problematic due to neutralizing antibodies.

4. Approaches to improving the kinetics of Ad-delivered genes and gene products

The disadvantageous kinetics of Ad vector-delivered genes and gene products for gene therapy applications results from their immunogenic toxicities. All the potential approaches to improving the kinetics should be based on attenuating the immunologic interaction between Ad vectors and hosts, so as to extend the persistence of the virus in the blood, reduce the accumulation of the virus and transgene expression in the liver, prolong circulational or local transgene expression in the organ/tissue of interest, and make readministration possible.

4.1. Viral genome deletion

Ad vectors have been extensively engineered to reduce their immunogenicity. First-generation Ad vectors were deleted for only one or two viral early genes (E1 and E3). Cells transduced with these vectors expressed other Ad genes at low levels, inducing strong cytotoxic T-cell responses that rapidly eliminated transgene expression. Second-generation vectors that contain additional deletions in other early genes (E2 and/or E4) have shown reduced toxicity profiles compared to first-generation Ad vectors due to the decreased Ad protein synthesis in transduced cells [49–51]. However, the remaining viral gene expression still induces the T-cell response, which is difficult to overcome.

Progress has been made in reducing T-cell responses against viral gene products expressed by transduced cells, by engineering "helper-dependent" (HD) or "gutless" or "gutted" Ad vectors, from which all viral genes are deleted except the inverted terminal repeats (ITR) sequences at the two ends and the packaging signal of the Ad genome. The HD Ad vectors are produced with a helper Ad that provides in-trans the necessary viral proteins required for replication and packaging of the HD vector (Fig. 4). This advance has improved the prospects of Ad vectors for long-term gene transfer [52]. Several application experiments have shown that the HD Ad vectors have facilitated life-long phenotypic correction in mouse models with negligible toxicity. For example, in a mouse model of hyperlipidemia, a defect correction was observed for 2.5 years with a single injection of an HD Ad vector [53]. In another mouse model of hemophilia, expression of human factor VIII was sustained for longer than 9 months [54]. However, in canine models of hemophilia A or B, only transient phenotypic correction, and in some of those cases only partial correction, was observed with no detectable toxicity using an HD Ad vector [55,56], while the same group of researchers achieved long-term phenotypic correction in a mouse hemophilia B model without toxicity by a single injection of an HD Ad vector [57]. Though the discrepancy of transgene persistence by HD Ad vectors between mouse and large animal models still needs to be elucidated, it is clear that immunogenic toxicities induced by HD Ad vectors are greatly reduced.

Compared with early generation Ad vectors, yields of HD Ad vector production need to be increased, and caution should be taken to decontaminate helper Ad. Some advances have been achieved in these aspects. Sakhuja et al. [58] developed an optimized HD Ad vector production system by generating a novel producer cell line, PERC6-Cre, which was adapted to serum-free suspension culture for bioreactor mass

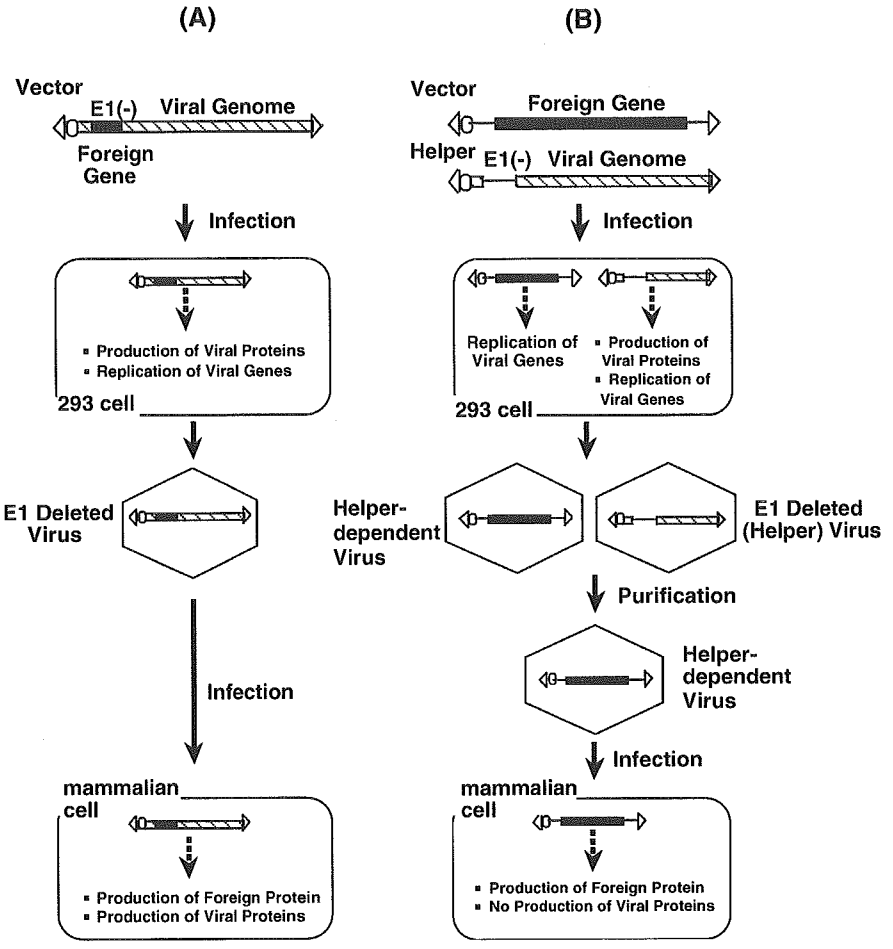

Fig. 4. Propagation diagrams of the first-generation Ad and helper-dependent Ad vectors. (A) The first-generation Ad vectors are produced in E1-transcomplementing cell lines, such as 293, 911, or PER.C6 cells. (B) Helper-dependent (HD) Ad viruses are propagated with the viral proteins provided by helper Ad viruses. To reduce the production of the helper virus, a Cre/loxP recombination system is generally utilized to excise the packaging signal from the helper virus genome [59].

production of HD Ad vectors. However, they also indicated that using the existing Cre/loxP technology to excise the packaging signal from the helper virus genome, which was originally developed by Graham and colleagues [59], could not completely eliminate the helper virus from HD Ad preparations.

With the advantages of reduced toxicity, larger packaging capacity for foreign genes of up to 36 kb, and possible persistent transgene expression, HD Ad vectors remain a powerful tool for gene therapy, though the humoral response against incoming capsid proteins shortly after administration remains a major challenge.

4.2. Modulation of the viral tropism

The broad tropism of Ad, on one hand, leads to unwanted vector uptake by many different cell types in multiple organs when the vectors are delivered systemically. Even the local delivery of Ad vectors can lead to leakage and dissemination to other tissue, resulting in toxic effects on distal sites, most notably the liver [60–62]. On the other hand, important types of target tissues are refractory to Ad infection due to CAR scarcity; these include primary tumor cells [63–66], mature skeletal muscle [67], endothelial [68,69], smooth muscle [68,69], differentiated airway epithe-

lial [70–72], lymphocytes [69,73,74], fibroblasts [68,69,75], hematopoietic cells [76] and monocyte-derived dendritic cells [77,78] and require an escalating dose of vector in order to achieve efficient gene transfer. This in turn increases vector-associated immunogenic toxicities. Hence, lack of Ad vector specificity is directly linked to the induction of massive systemic immune responses. Furthermore, localizing gene transfer by Ad vectors to specific cell types is likely to reduce immunogenic toxicities by allowing lower does to be administered. Therefore, there is a strong rationale for the development of tropism-modulated Ad vectors of enhanced specificity and gene transfer efficiency. In recent years, there have been significant efforts to improve Ad transduction efficiency to targets that are resistant to Ad infection due to CAR deficiency [79,80]. Ad vectors with the native tropism completely ablated have also been successfully developed [42,81].

4.2.1. Increasing transduction of target cells by bispecific conjugates

Douglas et al. [82] first reported the bispecific conjugate-based approach. They conjugated folate to the neutralizing Fab fragment of an anti-fiber monoclonal antibody (mAb). This Fab–folate conjugate was complexed with an Ad vector and was shown to redirect the Ad infection of target cells via the folate receptor at a high efficiency. Furthermore, when complexed with an Ad vector carrying the gene for herpes simplex virus thymidine kinase, the Fab–folate conjugate mediated the specific killing of cells that overexpress the folate receptor [82]. After that, the Fab fragment of the anti-fiber monoclonal antibody has been utilized to conjugate with several other ligands. For example, the Fab has been conjugated with (1) basic fibroblast growth factor (FGF2) to target various cells [83], including Kaposi's sarcoma cell lines [84], and ovarian cancer cells [85–87]; (2) mAb against the epidermal growth factor receptor (EGFR) to target glioma cells [64] and squamous cell carcinoma [88]; (3) an anti-CD40 mAb fragment to target dendritic cells [77]; (4) anti-angiotensin converting enzyme (ACE) mAb to target pulmonary endothelial cells [89]; (5) and an Hc fragment of tetanus toxin to target neuronal cells [90]. Theoretically, in this approach, any conjugates with one component directed against the Ad capsid and the second component directed against the cell surface protein can be applied to increase transduction of the target cells. The component directed against the Ad capsid can be the neutralizing Fab fragment of an anti-fiber monoclonal antibody as described above, a neutralizing anti-Ad knob single-chain antibody (scFV) [91–95] or the extracellular domain of CAR [96,97]. The targeting cell-binding moiety can either be natural molecules or man-made peptides identified by phage display technique [92,98,99].

4.2.2. Increasing transduction of target cells by genetic modification of the fiber

Since the fiber stretches out from the capsid and plays a central role for Ad binding to the native receptors, many attempts have focused on genetic modifications of the fiber. This approach can be divided into two main sub-approaches.

One is to incorporate ligands into the fiber knob. In order not to destroy the fiber trimerization, and to facilitate the ligands to access their cognate receptor, the HI loop and C-terminal of the Ad fiber knob have been found to be most appropriate to accommodate the foreign ligands [100,101]. We and other groups showed that Ad vectors containing the RGD motif in the HI loop greatly increased by as much as 3 orders the efficiency of gene delivery to a variety of CAR-deficient cells including primary and established ovarian cancer cells [102,103], squamous cell carcinoma [104], leukemia [105,106], rhabdomyosarcoma [107], dendritic cells [108,109], glioma [94,105], pancreatic cancer cells and primary human endothelia cells [103]. These results indicate that the integrin family could be very efficient mediators for expanding the native tropism to various CAR-deficient cells by RGD-modified Ad vectors. Currently, the RGD-modified Ad vector is being tested in a phase I clinical trial of ovarian cancer and recurrent cancer of the oral cavity and oropharynx [80]. Besides the RGD motif, there are reports of inserting the peptide SIGYPLP (Ser-Ile-Gly-Tyr-Pro-Leu-Pro), which was discovered by phage display to show high affinity to vascular endothelial cells [92], in the HI loop to increase transduction of vascular endothelial cells [110] and cancer cells [111]. In terms of the incorporation of foreign peptide into the C-terminal of fiber knob, we and another group found that a peptide containing seven lysine residues could be

inserted to increase transduction efficiency to a variety of CAR-deficient cells [69,112].

Another sub-approach is fiber-pseudotyping. Since Ads that belong to subgroup B, such as Ad11, Ad14, Ad16, Ad21, Ad35, and Ad50, recognize CD46 as the primary cellular receptors [113,114], fiber (knob, or knob and shaft) substitution could alter the tropism of Ad5 vectors. This strategy was first reported by Gall et al. [115]. They constructed a chimeric Ad5 vector by replacing the Ad5 fiber gene with the fiber gene from Ad7 (although Ad7 belongs to subgroup B, its receptor has not been identified), and found altered tropism to Ad5 vectors. Shayakhmetov et al. [76] constructed an Ad5 vector with a chimeric fiber (Ad5 tail/Ad35 shaft/Ad35 knob) and showed increased transduction of CD34+ cells relative to the unmodified Ad5 vector. Also, the same group incorporated the Ad11 fiber to Ad5 and found enhanced infection of human hematopoietic progenitor cells [116]. 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 [117] and an 150-fold increase in gene transfer to cultured synoviocytes as compared to Ad5 [118]. Replacement of only the knob domain of the fiber can also alter viral tropism. Stevenson et al. [119] demonstrated that replacement of the Ad5 fiber knob with the Ad3 fiber knob (which, though it also belongs to subgroup B, has been shown not to use CD46 as a high-affinity attachment receptor [113,114,120]) improved gene delivery to human fibroblasts and head and neck cancer cells when compared to unmodified Ad5. Takayama et al. [121] generated a dual-knob mosaic Ad virus by incorporating both Ad5 and Ad3 knobs in the same particle, which displayed infectivity enhancement and tropism expansion by utilizing either receptor, CAR or the Ad3 receptor, for virus attachment to cells.

In addition to the approaches at the level of transduction, increasing transduction of the target cells can also be achieved to some extent at the level of transcription by using cell-specific promoters. Furthermore, combining transductional and transcriptional targeting seems to be an attractive strategy to enhance the targeting effect of Ad vectors. Reynolds et al. [122] reported that in an Ad vector, the combination of transductional targeting by linking the Fab fragment of an anti-Ad5 knob antibody to the anti-ACE (pulmonary endothelial marker) monoclonal antibody mAb, and an endothelial-specific promoter (flt-1) resulted in a synergistic, 300,000-fold improvement in the selectivity of transgene expression for the lung versus the usual site of vector sequestration, the liver. Barnett et al. [123] obtained great synergistic targeting effect in cancer cells using a similar dual targeting strategy with the target molecule being epidermal growth factor receptor (EGFR), which is overexpressed on many tumor cells; the specific promoter was the osteocalcin gene 2 promoter, which has specificity for osteoblasts and osteoblastic metastatic lesions. Nicklin et al. [111] also observed the synergistic targeting effect in certain cancer cells by combining transductional targeting (incorporating the SIGYPLP peptide into the fiber knob) with transcriptional targeting (via the FLT-1 promoter).

4.2.3. Ablation of the native tropism

Though the above tropism-modified Ad vectors could greatly improve transduction efficiency to many CAR-deficiency cells, when systemically administered, sometimes vector dissemination, resulting in liver accumulation, is still unavoidable. To create a strictly targeted Ad vector, two basic requirements are thought to be necessary: interaction of Ad with its native receptors must be completely removed and novel tissue-specific ligands must be added to the virus capsid (Fig. 5).

The capsid proteins determine the tropism of Ad. The fact that Ad5 uses multiple receptors such as CAR, αv integrin and heparan sulfate to transduce various cells implies that the Ad5 capsid must be multi-engineered to abolish its native tropism. Several groups including us have shown that vectors with the ablation of only CAR-binding, i.e., vectors in which the AB, DE, or FG loop of the fiber knob was mutated, do not change the systemic gene-transfer properties [124–127]. Vectors with the ablation of only αv-integrin-binding also show similar or slightly decreased liver transduction compared with wild-type Ad vectors [126]. Furthermore, the length [128–130] and the KKTK motif of the fiber shaft [81] have been reported to influence Ad5-mediated in vivo gene transfer. We supposed that Ad5 tropism would be determined by at least three factors: the fiber knob, the fiber shaft and the RGD motif at the penton base. Thereby, we developed a triple-mutant Ad5 vector by

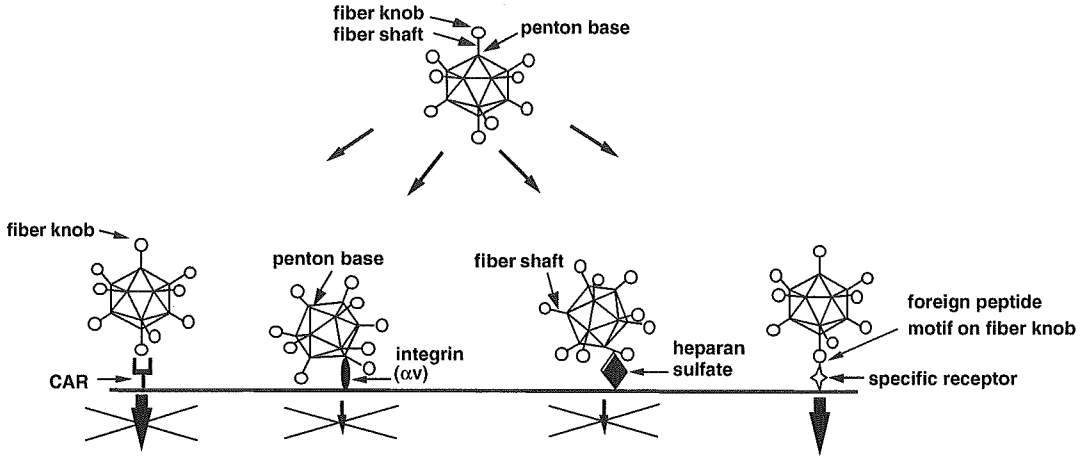

Fig. 5. Schematic diagram of targeted Ad vectors with the original tropism ablated. With the triple mutations in the fiber knob, fiber shaft and penton base, the CAR-, αv intergrin- and heparan sulfate-binding activities of the Ad viral capsid are completely ablated. Targeting effects can be achieved by either the bispecific conjugate or the foreign ligands incorporated into the fiber knob.

(1) mutating the fiber knob to ablate CAR-binding interaction, (2) replacing the Ad5 shaft with a shorter shaft from Ad35, which contains no KKTK motif to ablate binding with heparan sulfate, and (3) depleting the RGD motif at the penton base to ablate αv-integrin-binding interaction. As expected, this triple-capsid-mutant Ad5 vector exhibited little tropism to any organs (Fig. 6). Compared with the wild-type Ad5 vector, it showed 30,000-fold lower mouse liver transduction [42]. This indicates that to ablate the original tropism of Ad vectors, all three parts of the capsid (fiber knob, shaft and penton base) associated with the original tropism should be simultaneously blocked. Smith et al. [81] utilized a similar strategy and reached the same conclusion as ours.

Due to the multiple mutation of the capsid, the triple-mutant Ad vectors could not be produced by the original protocol using 293 cells. To support the propagation of our triple-mutant Ad5 vector, we generated a mutant 293 cell line stably expressing wild-type Ad5 fiber protein (Fiber-293 cells). It is possible to produce this mutant Ad vector to high titer using Fiber-293 cells. Furthermore, for the convenient display of foreign ligands, both the HI loop and C-terminal region of the fiber knob and the region of the RGD motif of the penton base were designed to have unique restriction sites. Therefore, by using our simple in vitro ligation method, the targeting ligands can easily be displayed in the capsid of our triple-mutation Ad vector [42]. We suggest that our triple-mutation Ad vector provide for a platform for future targeted Ad vector development. Future efforts should be directed into exploring novel ligands for specific tissue targeting.

Our triple-mutant Ad vector described here should be easily combined with other approaches such as transductional targeting, transcriptional targeting and even deletion of viral genomes to create less immunogenic vectors. Such combination will no

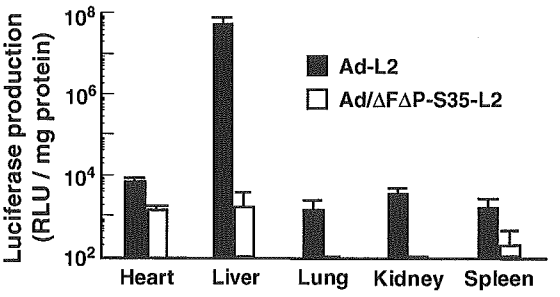

Fig. 6. Biodistribution of a transgene product (luciferase) in mice after the systemic administration of Ad-L2 (conventional Ad vectors) or Ad/ΔFΔP-S35-L2 (with triple mutations in the regions of fiber knob, fiber shaft and penton base). Ad-L2 or Ad/ΔFΔP-S35-L2 (3.0×10^{10} VP) were intravenously injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were isolated, and luciferase production was measured by luminescent assay. All data represent the mean±S.E. of five mice. The data in this figure were published in our previous paper [42].

doubt improve the kinetics of Ad vectors to better meet the requirements of clinical trial.

4.3. Cationic liposome complexation or PEGylation

The most straightforward approach for circumventing Ad-induced immunogenic toxicities has been the use of cationic liposome and polyethylene glycol (PEG) to shield the vectors from the host immune system.

Cationic liposomes have shown promise as a gene delivery tool for plasmid DNA due to their simplicity, safety and efficiency in some tissues [131]. Several groups have observed that cationic liposome-conjugated Ad vectors greatly increase gene transduction efficiency to a variety of cell and tissue types that are especially resistant to Ad infection, including vascular tissue [132], human smooth muscle cells [133], airway epithelia [134] and human glioma cells [135]. Fasbender et al. [134] suggested that viral binding was dependent on an electrostatic interaction with the cell surface, that viral entry did not require an interaction of the Ad fiber protein with the cell surface, and that cationic liposome-conjugated Ad vectors entered cells via a pathway different from that utilized by Ad alone. Use of a more efficient delivery system could allow a smaller dose of Ad vector to be administered for therapeutic effects, thereby decreasing the total immune response. Yoshida's group noted that mice injected with cationic liposome-conjugated Ad vectors produced fewer anti-Ad antibodies compared with an equivalent dose of unconjugated Ad vectors, and cationic liposome-conjugated Ad vectors were less susceptible to inactivation by neutralizing antibodies than Ad vectors alone [136,137]. The increased transduction efficiency, reduced antigenicity, and attenuated susceptibility to neutralizing antibodies might be beneficially multiplied for redosing.

Complexation with PEG (PEGylation) is frequently used in pharmaceutic preparations to provide a hydrophilic coat and to increase blood persistence of therapeutic proteins such as erythropoietin (EPO), granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) etc. [138–140]. Covalent attachment of PEG to the surface of the Ad vector might prolong persistence in the blood and circumvent neutralization of Ad vectors by antibodies [141]. There is a report that PEGylated Ad vectors exhibited a 4-fold slower clearance rate than the non-PEGylated Ad vectors [38]. Furthermore, coupling of PEG to the viral capsids attenuates the ability of the vector to infect antigen presenting cells, thereby reducing inflammatory responses. Animals administered with the PEGylated Ad vectors exhibited reduced levels of both cell-mediated and humoral immune responses, resulting in significant gene expression upon readministration of unmodified Ad vectors in the lung [141,142]. However, PEGylation might lead to loss of infectivity [38]. The activated PEG reacts preferentially with the ε-amino terminal of lysine residues on the capsid, including the hexon, fiber and penton base, which might lead to decreased infectivity. Experimental optimization of the PEGylation reaction so that PEG occupies only 70 to 80% of the available sites on virus capsid proteins shielded vectors from antibody neutralization and retained viral infectivity [143,144]. Nonetheless, compared with local readministration, the results of systemic readministration of the PEGylated Ad vectors were less encouraging. Levels of transgene expression achieved by systemic readministration of the PEGylated Ad vectors were on the order of 1000- to 10,000-fold lower than that seen in animals carrying no neutralizing antibodies against Ad; these levels were also significantly lower than those observed with readministration of these modified vectors in the lung [142]. Thus, the exact nature of the immune response against the PEG modified Ad must be characterized further.

4.4. Immune intervention

The success of long-term gene therapy by Ad vectors depends on finding ways to avoid/attenuate the induction of immune responses to both the vector and the transgene product. Some advances have been made in the development of novel strategies to disrupt or modulate immune responses in various animal models; these approaches include inhibiting cell-mediated immune responses to prolong transgene expression, and inhibiting humoral immune responses to permit readministration of the vector.

One straightforward approach is to disrupt the inflammatory immune response by inhibition of NF κB activation, thereby blocking release of inflammatory cytokines like TNF-α and IL-6 [145,146]; other relatively simple approaches include pretreatment by

or coadministration of anti-inflammatory drugs such as steroids [147–150]. Lieber et al. [145] obtained persistent Ad-delivered hAAT [human alpha (1)-antitrypsin] expression in bcl-2 transgenic mice for longer than 3 months by coadministrating an Ad vector expressing an IκBα supersuppressor to unravel the role of virus-induced NF κB activation [151,152]. They found that to confer vector persistence, simultaneous expression of bcl-2, an antiapoptotic protein [153], was required to block virus-induced apoptosis, while NF κB protection was inactivated by the IκBα supersuppressor. Kolb et al. [147] investigated the use of topical corticosteroids in improving gene expression after repeated injection of Ad vectors into mouse lungs. They showed that budesonide given around exposure to Ad to the lung significantly helped maintain high levels of the expressed transgene protein in bronchoalveolar lavage fluid after as many as four consecutive injections of virus at 2-week intervals. Furthermore, they observed that the improved transgene expression in budesonide-treated animals was associated with a reduction, but not prevention of neutralizing antiviral antibodies.

Another approach is the use of immunosuppressive drugs to inhibit cell-mediated immune functions, just as they are used in clinical trials for organ transplantation. The central role of CD4+ T cells in the activation of cellular and humoral immune response has focused immunosuppressive strategies towards blockade of costimulatory molecules. Recombinant murine CTLA4Ig (an inhibitor of the CD28/B7 pathway) and the anti-CD40 ligand antibody block costimulatory interactions between T cells and antigen presenting cells. Blocking of costimulatory signals has been shown to inhibit T-cell activation in several animal models of Ad vector gene therapy. Kay et al. [154] observed that when the anti-CD40 ligand and recombinant murine CTLA4Ig were coadministered around the time of primary Ad vector administration, Ad-mediated gene expression was maintained up to 1 year in mouse livers, and persistent secondary Ad-mediated gene expression lasted for at least 200-300 days even after the immunosuppressive effects of these agents were no longer present. However, neither agent alone allowed transduction after secondary vector administration. Jooss et al. [155] described a strategy that aimed to inhibit CD4+ T cell activation by transiently administering CTLA4Ig at the time an E1-deleted Ad vector is administered to the liver or lung. In the lung, CTLA4Ig treatment significantly blocked the formation of neutralizing antibodies, allowing efficient readministration of the vector, whereas transgene expression was only moderately prolonged. In contrast, CTLA4Ig did not suppress neutralizing antibody formation in the context of liver gene therapy, but resulted in more stable gene expression. These observations suggest that it may be possible to obtain persistence as well as secondary Ad-mediated gene transfer with transient inhibition of the CD28/B7 pathway at the time of virus instillation, especially in a local route. However, immune intervention in systemic Ad administration seems to be much complex. Moreover, this immunomodulation might compromise the host immune response. Thus, the clinical utility is uncertain.

4.5. Optimization of in-cis acting elements

As discussed in the previous section, the immunogenic toxicities of Ad vectors can lead to clearance of the transduced cells and only transient transgene expression. The severity of the immunogenic toxicities is intimately connected with vector dose. However, to achieve anatomically detectable and physiologically relevant levels of transgene expression, the number of Ad particles to be injected is always too large to avoid severe immunological side effects [156,157]. One simple but practical way to alleviate this kind of immunogenic toxicities is to improve the expression efficiency of each viral particle unit so that the number of Ad particles to be injected can be decreased.

Transgene expression can be modulated at both the transcriptional and post-transcriptional levels. We systematically investigated the ability of transcriptional regulatory elements [promoter, enhancer, intron and poly(A) sequences] and post-transcriptional regulatory elements to maximize the transgene expression efficiency from each Ad vector unit [158]. Our optimized Ad vector, Ad-WCMVL2, with an expression cassette containing the human CMV promoter/enhancer, intron A, the luciferase gene, the Woodchuck hepatitis virus post-transcriptional regulation element (WPRE), and bovine growth hormone (BGH) poly (A), showed more than 700-fold luciferase expression in mouse liver than the Ad vector, Ad-

CMVL1, with an expression cassette containing only the human CMV promoter/enhancer, the luciferase gene and BGH poly (A), when they were intravenously administered into the mouse [158]. It may be deduced that to express transgenes at a therapeutic level, the in-cis acting elements of our optimized Ad vector, Ad-WCMVL2, may decrease, by several orders of magnitude, the number of viral particles that must be injected as compared with Ad-CMVL1 with the expression cassette containing only the CMV promoter/enhancer and the BGH poly(A) as in-cis acting elements which are considered to be one of the most strongest in-cis acting element combination and are widely used in clinical trials and gene function studies [159]. It also means that immunogenic toxicities induced by Ad particles could be greatly alleviated by using the in-cis acting elements of Ad-WCMVL2 to construct therapeutic Ad vectors. Gerdes et al. [160] demonstrated that the murine CMV promoter was much stronger than the human CMV promoter in the context of Ad vectors in all the cell lines tested, including nonhuman primate and human cell lines. By using this murine CMV promoter, the investigators observed that the Ad vector dose required to achieve sufficient transduction could be reduced 100-fold and the cellular inflammation and viral cytotoxicity associated with the delivery of Ad vectors into the rat brain could be completely eliminated.

In the context of HD Ad vectors, the inclusion of a human centromeric region and a matrix attachment region as in-cis acting elements improved the maintenance of the Ad vector genome and the transgene expression level [57]. It can be concluded that even for the Ad vectors that have an extremely high transduction efficiency, the in-cis acting elements can be optimized to obtain maximized transgene expression.

4.6. Integrating Ad vectors

For replication-deficient Ad vectors, the existence of episomal Ad DNA presents the disadvantage of not integrating for long-term expression of the delivered foreign gene. In the case that stable genetic alternation needs to be maintained in dividing cells, integrating vectors are at present the tools of choice. Though integration is not a guarantee of stable transcription, since transgene expression from integrated vector genomes can be gradually silenced over time in some cases [161], at present the best way to get long-term transgene expression is to integrate the foreign gene into the host genome.

To incorporate integration machinery to Ad, a variety of hybrid vectors combining the highly efficient DNA delivery of Ad with the integrating machinery of adeno-associated viruses (AAV), retroviruses, and transposons have been emerging [162]. The unique biology of AAV has stimulated considerable efforts toward the development of AAV-based integrating Ad vectors. Such integrating Ad-AAV hybrid vectors devoid of all Ad viral genes were successfully developed by Lieber et al. [163] and Recchia et al. [164]. Lieber's hybrid Ad-AAV vectors displayed in vitro an integration frequency comparable to that reported for AAV vectors, and high transgene expression at a level comparable to the first-generation Ad vectors [163].

Baun's group did extensive studies exploiting the retrovirus integrating machinery to realize permanent Ad-delivered transgene expression. By flanking the transgene cassette in the Ad vector with the cis-acting elements from Moloney murine leukemia virus in a unique arrangement, they obtained high integration frequencies (between 5% and 11%) in rat spleen cells [165]. In consideration of the high transduction efficiency, this integration rate should be high enough to stably keep transgene expression at therapeutic levels for many gene therapy applications.

Kay and colleagues addressed the challenge of genome persistence by exploiting the promiscuous integration capabilities of the Sleeping Beauty (SB) transposon in Ad-mediated gene delivery [166]. SB transposon is a member of the Tc1/mariner superfamily of transposons, and undergoes cut-and-paste transposition through a DNA intermediate, mediated by the SB transposase [167]. By incorporating the SB integration machinery into an HD Ad vector containing human coagulation factor IX, therapeutic levels of human coagulation factor IX were sustained for more than 6 months in mice undergoing extensive liver proliferation [166].

However, at the current time there are great concerns about the risk of insertional mutagenesis due to random integration of vectors into the host genome. The recent clinical trial in France using

retroviral vectors in a gene therapy approach for X-linked severe combined immunodeficiency disease (X-SCID) had been taken as the milestone success in human gene therapy [168]. Unfortunately, two of the 11 patients treated during this X-SCID trial developed a leukemia-like disorder [169,170]. Now it is known that the cause of induced leukemia in X-SCID trial is attributed to the retrovirus vector integration in proximity to the LMO2 proto-oncogene promoter, which leads to aberrant transcription and expression of LMO2 [171]. Recent study suggests that the AAV vector preferentially integrates into active genes, though at present it is not clear if this will contribute to the risk of developing a malignancy [172]. Recchia's Ad/AAV hybrid vector system showed 35% of DNA integration was site-specific at the AAVS1 site in hepatoma cells, with one HD Ad vector expressing Rep78 and the second HD vector carrying a transgene flanked by AAV-ITRs [164]. However, excess Rep expression is toxic to the cells and may result in rearrangement of AAVS1 without transgene integration [173]. To avoid potential mutation risks of random integration, episomally replicating Ad vectors might be a potential alternative choice for long-term expression. Leblois et al. [174] inserted a loxP flanked Epstein-Barr virus (EBV) replicon into an Ad vector. Expression of Cre recombinase from a second Ad vector has been shown to release the transcription unit that could replicate coordinately during the cell cycle. Kreppel et al. [175] incorporated the episomally replicating machinery to an HD vector by utilizing the DNA recombinase FLPe to circularize the genome containing the EBV replicon, and obtained long-term transgene expression in proliferating cells. However, data of episomally replicating Ad vectors from in vivo studies are lacking.

4.7. Serotype switch or animal Ad vectors for readministration

In many gene therapy applications, lifelong expression might be needed, which would require readministration of vectors following the eventual loss of therapeutic transgene expression if the vectors have no integration or episomal replication machinery. However, readministration of an Ad vector will require the circumvention of the humoral immune response directed against the original vector capsid. Studies indicated that fiber-substituted Ad5 vectors containing fiber proteins of another serotype could not evade the humoral immune response against Ad5 [115], because hexon proteins of the capsid are the major targets of host-neutralizing antibodies in Ad5 infection [115,176,177]. This suggests that capsid partially modified Ad5 vectors do not meet the long-term need. Hence, the most practical approach to overcoming the obstacle of pre-existing antibodies is the use of alternative serotypes.

Morral et al. [178] observed in baboons that the hurdle of readministration due to the humoral response to an Ad5 vector was overcome by use of an Ad2 vector expressing hAAT. Their data further suggest that long-term expression of transgenes should be synergized by combining the reduced immunogenicity and toxicity of HD vectors with sequential delivery of vectors of different serotypes. However, it might be difficult to completely avoid cross-humoral response between Ad2 and Ad5 in all species including humans, as both Ads belong to the same subgroup and have high homogenicity. To address this issue, we and other groups have developed Ad35 vectors (subgroup B) [179–182] and have found that antibodies to Ad5 vector do not affect the transduction efficiency by Ad35 vector (Sakurai et al. unpublished observation, 181). Furthermore, while there is more than a 50% prevalence in adult humans of neutralizing antibodies to Ad5, less than 10% of individuals have anti-Ad35 neutralizing antibodies [181].

Since many humans are pre-exposed to human Ad, Ads from non-humans have been developed as vector systems for gene delivery. Mouse [183], avian [184], bovine [185], canine [186], porcine [187] and chimpanzee [188] Ad have been tested as vectors for various applications to overcome the barriers of pre-existing neutralizing antibodies. These animal-derived Ad vectors might be useful in evading humoral immune responses to human Ad5 for readministration. However, prior to their clinical application, safety issues such as oncogenicity should be addressed.

Readministration might be a solution to the transient transgene expression of Ad vectors when it is necessary. To balance the gain and loss in safety, efficacy and economy are the key points. The potential serotypes and animal origin Ads are limited.

Furthermore, this approach needs a complex set of clinical products.

5. Summary

Among all the vectors for gene delivery, no single one can meet all the requirements for all gene therapy applications. Similarly, for Ad vectors, no single approach can overcome the hurdle of immunogenic toxicities. It seems that HD vectors might serve as basis for combination with other approaches. It is clear that the ultimately ideal Ad vectors will have low or no immunogenic toxicities, and specifically transduce interested tissues/cells, with high, persistent and regulatable transgene expression. It is also clear that there is still a long way before research reaches this ideal.

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