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Helper-dependent adenoviral vectors

Nicola Brunetti-Pierri and Philip Ng

Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA

Abstract

Helper-dependent adenoviral (HDAd) vectors have several characteristics making them attractive for human gene therapy. These vectors are completely devoid of viral coding sequences and are able to mediate high efficiency transduction *in vivo* to direct high level transgene expression with negligible chronic toxicity. However, clinical translation is complicated by the dose-dependent acute toxic response following systemic vector injection. With a better understanding of vector-mediated toxicity and improved delivery methods, HDAds may emerge as an important vector for gene therapy of human diseases.

Introduction

Gene therapy vectors derived from the adenovirus (Ad) are the most often used in clinical trials [1]. The majority of these applications are for cancer treatment and very few are for non-cancer diseases [1]. First generation adenoviral (FGAd) vectors were rendered replication-deficient by the deletion of the viral early region 1 (E1). FGAd can efficiently transduce a wide variety of cell types from many different species independent of the cell cycle to direct high levels of transgene expression. However, low levels of viral gene expression from the vector backbone result in loss of transgene expression due to immune-mediated clearance of transduced cells. In contrast, helper-dependent adenoviral (HDAd) vectors, which are devoid of all viral sequences, are safer and more effective for disease applications requiring long-term expression of the therapeutic gene [2]. A multitude of small and large animal models of genetic disorders can be corrected effectively and long-term by HDAd vectors without signs of chronic toxicity [3]. HDAd can mediate high efficiency transduction, do not integrate in the host genome, and have a large cloning capacity of up to ~37 kb which allows for the delivery of whole genomic loci, multiple transgenes, and large *cis*-acting elements to enhance, prolong, and regulate transgene expression. This chapter will present the general features of the HDAd and will focus on recently developed applications for liver, lung, and brain gene therapy.

HDAd

HDAd are derived from the Ad, a non-enveloped icosahedral capsid containing a linear double-stranded DNA genome of ~30–40 kb. The Ad genome is flanked by inverted terminal repeats (ITRs) which are the only sequences required in *cis* for viral DNA replication. A *cis*-acting packaging (ψ) signal, required for encapsidation of the genome, is located near the left ITR (relative to the conventional map of Ad). The Ad genome can be divided into two sets of genes (Fig. 1): the early region genes (E1A, E1B, E2, E3, and E4) expressed before DNA replication and the late region genes (L1 to L5) expressed after initiation of DNA replication. The early region genes are expressed during viral infection and are involved in transcriptional regulation of the viral genome. The late region genes mostly encode virion structural proteins.

The first and most efficient method for generating HDAd is the Cre/loxP system [4] (Fig. 2). In this system the HDAd genome, constructed in a bacterial plasmid, contains: i) the ITRs and ψ signal, ii) the expression cassette of interest, and iii) stuffer DNA up to ~36 Kb required for efficient packaging [5, 6]. To convert the plasmid form of the HDAd genome into the viral form 293

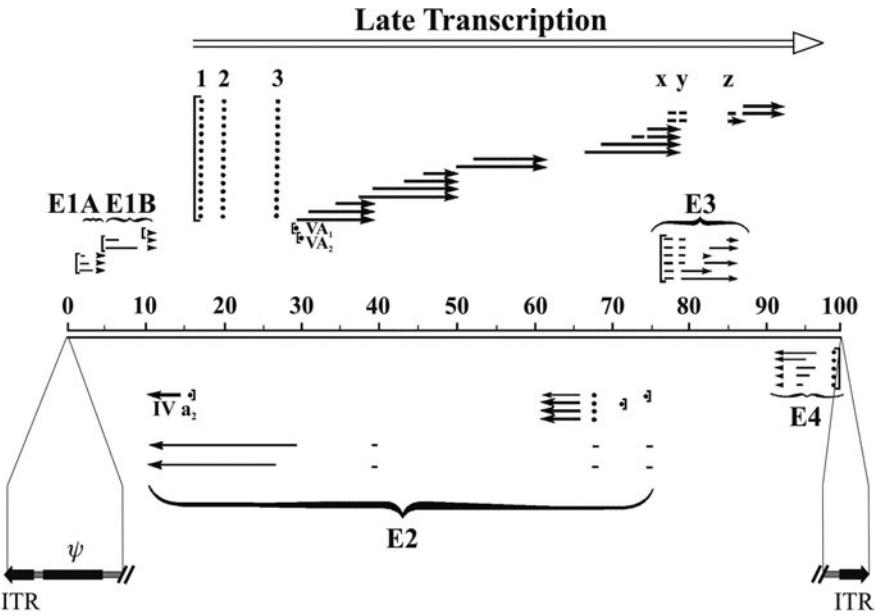

Figure 1. Transcription map of human adenovirus serotype 5. The 100 map unit (~36 kb) genome is divided into four early region transcription units, E1–E4, and five families of late mRNA, L1–L5, which are alternative splice products of a common late transcript expressed from the major late promoter located at 16 map units. Four smaller transcripts, pIX, IVa, and VA RNA's I and II, are also produced. The 103 bp inverted terminal repeats (ITRs) are located at the termini of the genome and are involved in viral DNA replication, and the packaging signal (ψ) located from nucleotides 190 to 380 at the left end is involved in packaging of the genome into virion capsids.

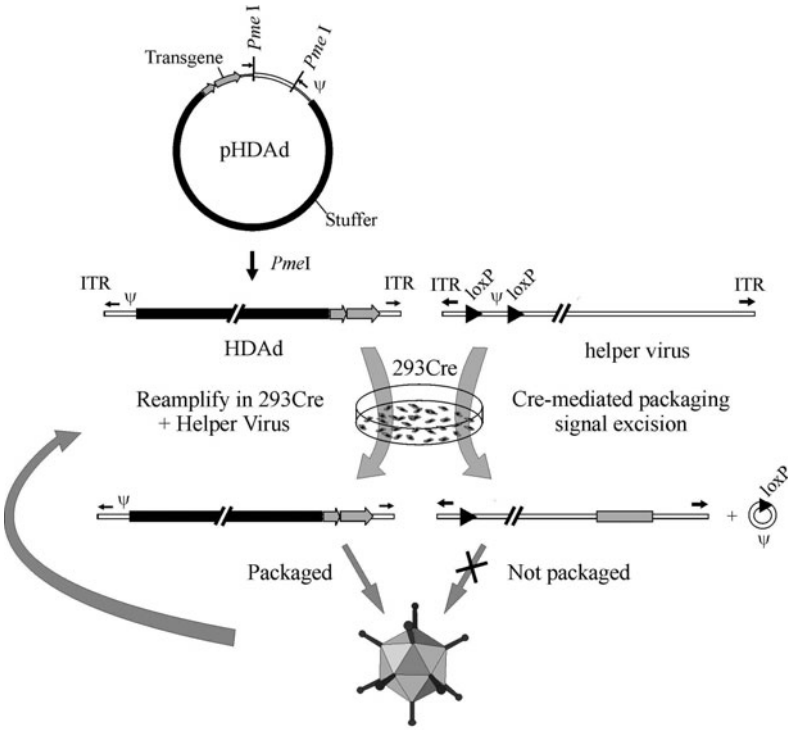

Figure 2. The Cre/loxP system for generating HDAds. The HDAd contains only ~500 bp of *cis*-acting Ad sequences required for DNA replication (ITRs) and packaging (ψ), the remainder of the genome consists of the desired transgene and non-Ad *stuffer* sequences. The HDAd genome is constructed as a bacterial plasmid (pHDAd) and is liberated by restriction enzyme digestion (e.g., *Pme*I). To rescue the HDAd, the liberated genome is transfected into 293 cells expressing Cre and infected with a helper virus bearing a packaging signal (ψ) flanked by loxP sites. Cre-mediated excision of ψ renders the helper virus genome unpackageable, but still able to provide all of the necessary *trans*-acting factors for propagation of the HDAd. The titer of the HDAd is increased by serial coinfections of 293Cre cells with the HDAd and the helper virus.

cells expressing Cre are transfected with the linearized HDAd genome and subsequently infected with the helper virus. The helper virus is a FGAd bearing a packaging signal flanked by loxP sites and following infection of 293Cre cells, the packaging signal is excised from the helper viral genome by Cre-mediated site-specific recombination between the loxP sites. This renders the helper viral genome unpackageable but still able to undergo DNA replication and thus *trans*-complement the replication and encapsidation of the HDAd genome. The production of large quantities of HDAd vectors with extremely low levels of helper virus contamination can be obtained through a rapid and efficient production method which makes the preparation of large vector stocks possible for large animal experiments and potentially for human applications [7].

In vivo studies with HDAd

As of this writing, numerous examples of *in vivo* HDAd-mediated gene transfer through different routes of administration (intravenous, intramuscular, brain and intratumoral injection, airway administration) in various small and large animal disease models have been reported. The purpose of this chapter is not to provide a comprehensive review of all of these studies. Instead, recent examples of particular significance or interest are described.

Liver directed gene therapy

The liver is a very attractive target for gene therapy because it is the affected organ in many genetic and acquired diseases and it can be used as a factory organ for systemic delivery through vascular circulation of vector-encoded therapeutic proteins. To date numerous examples of *in vivo* liver-directed gene therapy using HDAd in several monogenic disease models have been reported. In general, all these studies have demonstrated long-term phenotypic correction in the absence of chronic toxicity thus supporting the potential of HDAd for clinical applications [8, 9]. Importantly, these results have also been recapitulated in clinically relevant large animal models [10–14]. HDAd-mediated hepatocyte transduction can be exploited for numerous diseases beyond monogenic disorders. An interesting application has been reported for the treatment of Type 1 diabetes mellitus. In this study, two HDAds, one expressing *Neurod1* (a transcription factor expressed in developing and adult β-cells of the pancreas), and the other expressing *betacellulin* (a β-cell growth factor), co-injected systemically into diabetic mice, resulted in the formation, within the liver, of cell clusters exhibiting immunohistochemical and ultrastructural properties of the pancreatic islets [15]. Remarkably, the diabetic mice also showed a normalization of glucose levels.

In liver-directed approaches, HDAds expressing short hairpin RNA (shRNA) to silence specific target genes have also been used. For example, HDAd-driven expression of shRNA to the specific mouse genes resulted in approximately 75–90% silencing [16, 17] and in a mouse model of obesity and Type 2 diabetes (*db/db* mice) silencing of the transcription factor sterol regulatory element-binding protein-1c (SREBP1), which is upregulated in obese mice, resulted in a reduction in the body weight [16]. These initial studies could pave the way to a multitude of applications directed at silencing of specific genes for the treatment of a variety of genetic and acquired disorders. Interestingly, in contrast with previous reports showing severe toxicity and lethality following administration of AAV encoding shRNA [18], the HDAd expressing shRNA was clinically well tolerated in mice with only mild pathological and biochemical signs of hepatotoxicity [16, 17]. Moreover, saturation of the exportin-5 pathway, which shuttles cellular micro-RNA (miRNA) from the nucleus to the cytoplasm, was found in the case of AAV [18] and was

thought to be involved in the observed toxicity. In contrast, saturation of the exportin-5 pathway was not seen with HDAd expressing shRNA [17].

Recent studies have uncovered the opportunity to treat autoimmune disorders by expressing functional therapeutic protein into hepatocytes to induce tolerance to a specific protein [19–21]. Hepatic expression of a brain protein, for example, was found to be protective against the neuroinflammatory disease in a mouse model of multiple sclerosis [22]. The suppression of autoimmunity from transgene expression in the liver suggests that the introduction of antigens to the liver may have potential as a preventative or therapeutic intervention against autoimmune disease. Given their high efficiency of hepatic transduction and the ability to drive long-term expression, HDAd would be well suited for this type of application.

A major problem with HDAd-mediated liver directed gene therapy which is preventing various therapeutic strategies to be translated into the clinic is the acute toxicity. High vector doses are required to achieve efficient hepatic transduction following systemic intravascular delivery because of a nonlinear dose response. Kupffer cells of the liver [23, 24], and antibodies both specific and nonspecific for Ad [23, 24] are involved in this nonlinear response. Unfortunately, systemic injection of high vector doses results in a potentially lethal inflammatory response secondary to the activation of the innate immunity and to the interactions with multiple cell types and bloodborne factors. The interaction with red blood cells appears to be particularly important as the majority of the Ad particles of serotype 5 are sequestered by human erythrocytes preventing liver infection [25–27]. Intravenous administration of Ad vectors also results in rapid recruitment of neutrophils in blood and peripheral tissues leading to acute liver inflammation and injury [28, 29], thrombocytopenia [14, 29–31], and widespread transduction of a large number of various other cell types (e.g., endothelium, spleen, lung, etc.). All these interactions play an important role in the activation of the toxic response. Furthermore, several blood factors interact with the Ad particles including proteins of the classical and alternative complement pathways [32–34] and several vitamin K-dependent serine proteases such as factors VII, IX, X, and protein C [35–37]. The interactions with these factors also play an important role in the transduction of target tissues because Ad5 hexon has high-affinity for human coagulation Factor X which facilitate virus entry into hepatocytes [37, 38].

Several groups have investigated various strategies to overcome the threshold to hepatocyte transduction and the obstacle of the acute toxicity. Because the severity of the acute response is dose-dependent, some of these approaches are aimed at preferential targeting of the vector to the liver thereby allowing the use of lower vector doses. For example, injection of HDAd directly into the surgically isolated liver of nonhuman primates was shown to achieve higher efficiency hepatic transduction with reduced systemic vector dissemination, and stable, multi-year transgene expression without chronic toxicity [11]. An alternative, minimally invasive, and clinically more attractive method to deliv-

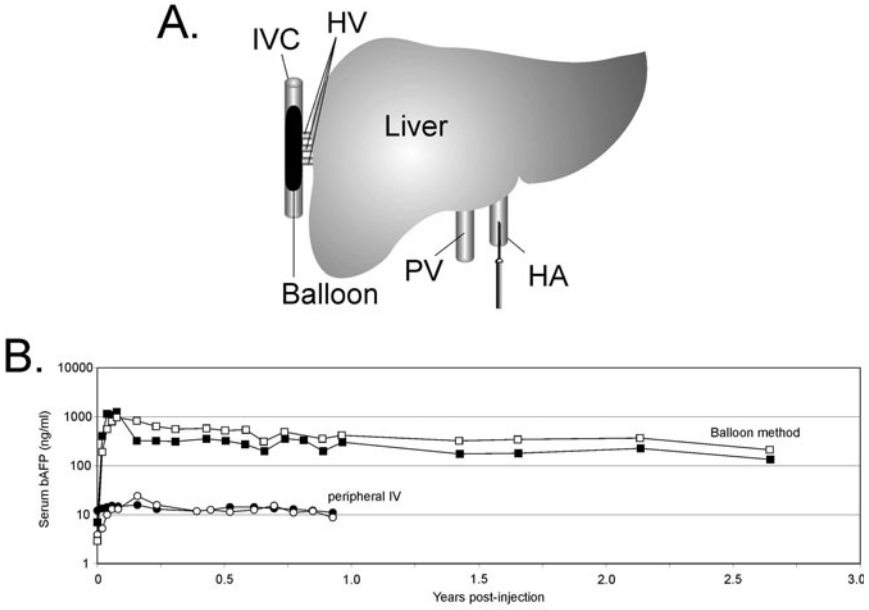

Figure 3. Minimally invasive method to achieve preferential liver transduction in nonhuman primates. (A) A sausage-shaped balloon catheter is positioned in the inferior vena cava (IVC) under fluoroscopic guidance. Inflation of the balloon results in hepatic venous outflow occlusion from the hepatic veins (HV). The HDAd is administered by injection through a percutaneously positioned hepatic artery (HA) catheter. (B). Serum levels of the reporter baboon α-fetoprotein (bAFP) following administration of 3×10^{10} vp/kg of a HDAd expressing bAFP into baboons using the balloon method described above (squares) or by simple peripheral intravenous injection (circles). The balloon method of vector delivery yielded up to 80-fold higher level of transgene expression compared to peripheral intravenous injection of vector, and transgene expression persisted at high levels for at least 2.5 years. Adapted from [13].

er HDAd preferentially to the liver has been developed using balloon occlusion catheters (Fig. 3) [10, 13].

Other strategies such as *masking* the viral capsid through liposome encapsidation [39] or PEGylation [40, 41] seem to attenuate the acute inflammatory response. Given the multiple factors involved, it appears more difficult to manipulate the innate immune response to systemic Ad injection. Nevertheless, a simple approach using pre-treatment with anti-inflammatory glucocorticoids (dexamethasone) before Ad administration, has been shown to significantly reduce Ad-induced acute responses, at least in mice [42].

Gene therapy for cystic fibrosis

The lung is an attractive target for gene transfer with the goal of treating cystic fibrosis (CF), one of the most common genetic disorders due to recessive

mutations in the cystic fibrosis transmembrane conductance regulatory (CFTR) gene. Several CF gene therapy clinical trials have been conducted [1] but no single class of gene therapy vector or vector delivery strategy has yet emerged as obviously superior and the results to date have been disappointing.

FGAd, extensively studied for CF gene therapy, have a number of serious shortcomings. First, pulmonary delivery of FGAd is inefficient because the cellular receptor for Ad (and other viral vectors) resides on the basolateral surface of the airway epithelial cells and the tight junctions prevent vector-receptor interactions required for transduction [43]. Second, pulmonary delivery of FGAd results in dose-dependent inflammation and pneumonia [44–48] beginning about 3 to 4 days post-administration and becoming progressively more severe before eventually resolving. This latter problem has been attributed to the expression of the viral genes of the FGAd vector backbone which are cytotoxic and cause an adaptive cellular immune response against the transduced cells resulting in loss of transgene expression and chronic toxicity [49, 50]. The first obstacle was addressed using molecules that disrupt the tight junctions which resulted in extensive Ad-mediated transduction of the proximal and distal airways (Figs 4A and B) and submucosal glands (Fig. 4C). The second obstacle was solved with the use of HDAd: while administration of FGAd results in pulmonary inflammation with focal peribronchial lymphocytic infiltrates and focal alveolar macrophages, the lungs of mice given HDAd are free of inflammation and indistinguishable from saline treated animals presumably because of the absence of viral gene expression from HDAd [51]. Moreover, the duration of HDAd-mediated pulmonary transgene expression persisted for at least 15 weeks [51]. The studies with HDAd have also indicated that the human cytokeratin 18 (K18) promoter is expressed, similarly to the mouse Cftr, in the epithelium of large airways and bronchioles and in submucosal glands with little expression in the alveoli [52]. In contrast to commonly used viral promoters, the K18 promoter is less likely to suffer host shut-off and could reduce immune stimulation resulting from inappropriate expression in

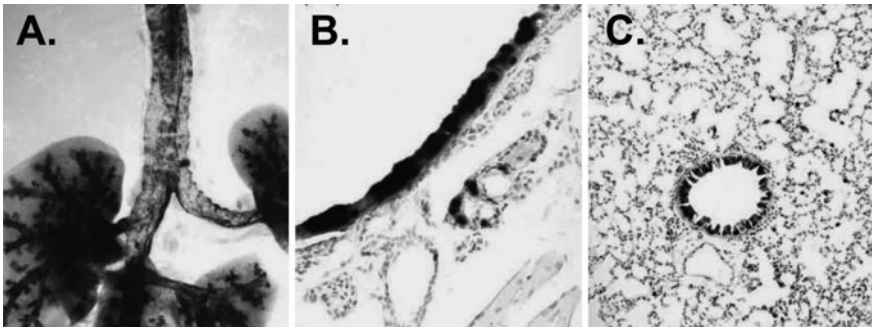

Figure 4. Airway transduction by HDAd. Epithelia transduction of the proximal and distal airway (A), trachea (B), and bronchiole (C) of mice 3 days post-intranasal administration of HDAd-K18LacZ. Blue areas represent HDAd transduced cells. From [51].

antigen presenting cells. The large cloning capacity of HDAd makes this vector ideal to accommodate the relatively large K18 control elements (4.1 kb) and the reporter or therapeutic cDNAs.

An HDAd vector bearing the human CFTR cDNA under the control of the K18 was also found to express properly localized CFTR in cultured cells and in the apical airway epithelia of mice following intranasal administration [53]. Importantly, this vector was also found to improve resistance to acute lung infection in CFTR knockout mice [53]. High efficiency transduction of the airway epithelium has also been demonstrated in a large animal model (rabbit) using an HDAd, formulated in 0.1% L-α-lysophosphatidylcholine (LPC) to open the tight junctions, and delivered by an intracorporeal nebulizing catheter called the AeroProbe (Trudell Medical International) to aerosolize material directly into the trachea and lungs [54]. Although high interlobular variation was present, the delivery of HDAd revealed exceedingly high and unprecedented transduction from the trachea to terminal bronchioles (Figs 5A–E). All rabbits, including those given only LPC as controls showed a transient decrease in dynamic lung compliance immediately following aerosol delivery. Fever and mild-to-moderate patchy pneumonia without edema were also observed. It is possible that LPC may have contributed to these effects which may be eliminated or minimized by optimizing the LPC and/or vector doses. Nevertheless, this study significantly demonstrated for the first time high efficiency transduction of the airway epithelium in a large animal which had previously been a major obstacle to CF gene therapy. This strategy has been applied to nonhuman primates and has yielded similar encouraging results [55]. A uniform vector distribution to all lung lobes was also achieved in the nonhuman primate model by targeting HDAd aerosolization individually into each lung lobe. This strategy resulted in an exceedingly high transduction efficiency to all lung lobes with negligible toxicity [56]. It should be pointed out that the aforementioned studies were performed in animal models with generally intact airways and that transduction will likely be reduced in the lungs affected by multiple bacterial colonizations and thick mucus such as the human CF lungs. Up to now efficacy of gene therapy has been only addressed in animal models with unaffected airways such as the CFTR knockout mice and the nonhuman primates. The recently developed pig model for CF could potentially provide a better model for assessing the efficacy of experimental treatments in the CF lung disease [57]. However, several strategies can be envisioned to address this obstacle in the clinical setting. For example, severely affected CF patients may undergo commonly employed regimens to clear their lungs before gene transfer. This could include inhaled antibiotics (such as tobramycin) and systemic intravenous anti-pseudomonal antibotics (such as aminoglycosides, beta lactams, fluoroquinolones), pulmonary treatment with mucolytic agents (such as pulmozyme), along with mechanical airway clearance to reduce the amount of mucus. Conducting gene transfer in CF patients with less affected lungs may be an alternative option, including the enrollment of younger CF patients with little or no lung disease. While somewhat contro-

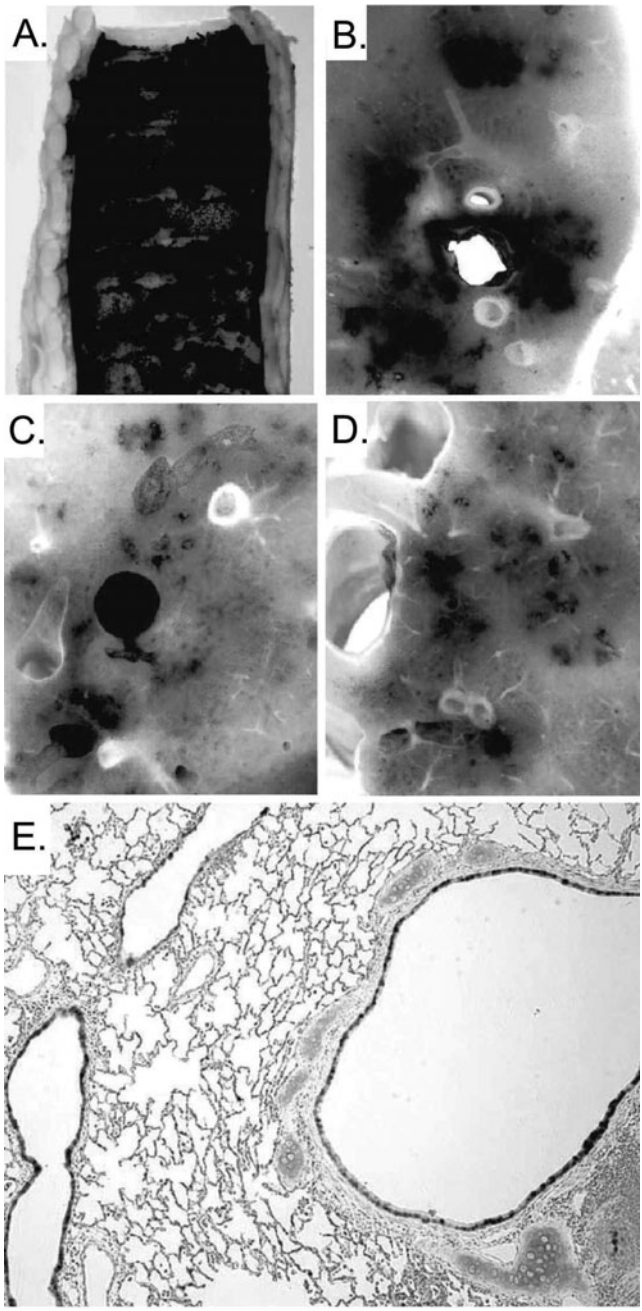

Figure 5. Pulmonary transduction in rabbits following AeroProbe-mediated intratracheal aerosolization of HDAd-K18LacZ formulated in 0.1% LPC. X-gal stained trachea (A), right upper lobe (B), left lower lobe (C), right lower lobe (C) and bronchus and bronchioles (D). Blue areas represent HDAd transduced cells. From [54].

versial, this is not without precedence. Indeed, in a recent clinical trial using AAV, CF patients as young as 12 years of age were enrolled [58]. In summary, while the thickened mucus remains a barrier for all gene transfer vectors (viral or nonviral) as well as for small molecule therapeutics, we do not believe it to be insurmountable, especially considering the low levels of gene transfer that may be required for CF phenotypic correct.

Brain gene therapy

The use of Ad vectors to deliver genes to the central nervous system (CNS) holds great promise for therapeutic applications. Because of their ability to infect post-mitotic cells, including cells of the CNS [59], and to mediate long-term transgene expression, Ad-based vectors are particularly attractive for these applications. Moreover, the delivery to CNS cells of anti-inflammatory genes is attractive for the treatment of inflammatory disorders such as multiple sclerosis. Unlike the rapid decline observed in transgene expression in peripheral organs following intravenous administration, FGAd-mediated transduction of adult brain cells leads to stable transgene expression [60, 61]. It is thought that FGAd-mediated long-term transgene expression occurs because the brain is relatively protected from the effects of the immune response, and in fact, Ad injection into the brain results in an ineffective T cell response against brain-transduced cells [62]. However, the immune system can respond to antigenic stimuli in the brain [63] and if a peripheral immune response against Ad is elicited after natural infection or vector readministration, loss of transgene expression and chronic inflammation are observed [64]. Interestingly, these problems are not seen when HDAd is used [64, 65]. For example, in naïve animals, the expression of β-galactosidase in the brain from FGAd or HDAd is sustained. However, in animals immunized prior to vector delivery, transgene expression is abolished in FGAd injected mice but not in the mice injected with HDAd. These results indicate that long-term (up to 1 year) HDAd mediated transgene expression in the brain occurs even in animals that had been immunized systemically against Ad before the delivery of HDAd into the brain. Therefore, HDAd vectors could turn out to be effective for gene therapy of chronic neurological disorders, even in patients who had been pre-exposed to Ad prior to gene therapy [66].

There are few examples of applications of HDAd for brain-directed gene therapy. Among these, encouraging results have been reported in a Huntington's disease mouse model showing a significant inhibition of Huntington protein aggregation following stereotactic injection into the striatum of a HDAd vector expressing a short hairpin RNA to silence the Huntington disease gene [67].

Besides the potential applications for neurodegenerative disorders, HDAd have also potential for the treatment of inflammatory diseases of the brain. Intrathecal administration of an HDAd expressing interleukin 4, for example,

has a protective role in mice against chronic or relapsing–remitting experimental autoimmune encephalomyelitis, modeling the most common clinical subtypes of multiple sclerosis [68]. Intrathecal HDAd administration is an attractive delivery method because the injection of viral vectors into the cisterna magna (in rodents) or trough lumbar puncture (in nonhuman primates) [69] allows viral vector transduction of neuroepithelial cells and delivery of transgene products to the whole CNS through the ventricular circulation.

Concluding remarks

HDAd possess many characteristics that make them attractive vectors for gene therapy of a wide variety of genetic and acquired diseases. For systemic delivery, the acute toxicity due to a multi-factorial reaction is the most significant obstacle currently hindering the clinical application of this otherwise promising technology. However, the potential of using HDAd for liver-directed gene therapy should not be dismissed but should instead proceed with caution considering the encouraging and compelling studies generated so far. Regardless of the multiple mechanisms involved, strategies to improve the efficiency of gene transfer using lower vector doses are clinically attractive because the acute toxic response is dose-dependent. Improvements in current technologies and development of novel strategies must be pursued to make HDAd gene therapy a clinical reality.

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