

Helper-dependent adenoviral vectors in experimental gene therapy*

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In the majority of potential applications gene therapy will require an effective transfer of a transgene *in vivo* resulting in high-level and long-term transgene expression, all in the absence of significant toxicity or inflammatory responses. The most efficient vehicles for delivery of foreign genes to the target tissues are modified adenoviruses. Adenoviral vectors of the first generation, despite the high infection efficacy, have an essential drawback: they induce strong immune response, which leads to short term expression of the transgene, and limits their usefulness in clinical trials. In contrast, helper-dependent adenoviral vectors (HdAd) lacking all viral coding sequences display only minimal immunogenicity and negligible side-effects, allowing for long-term transgene expression. Thus, HdAd vehicles have become the carrier of choice for adenoviral vector-mediated experimental gene therapy, effectively used in animal models for delivery of transgenes into the liver, skeletal muscle, myocardium or brain. Strong and long-lasting expression of therapeutic genes has allowed for successful treatment of dyslipidemias, muscular dystrophy, obesity, hemophilia, and diabetes. Additionally, the large cloning capacity of HdAd, up to 37 kb, facilitates the use of physiologically regulated, endogenous promoters, instead of artificial viral promoter sequences. This enables also generation of the single vectors expressing multiple genes, which can be potentially useful for treatment of polygenic diseases. In this review we characterize the basic features of HdAd vectors and describe some of their experimental and potential clinical applications.

Keywords: gene therapy, adenoviruses, helper-dependent adenoviral vectors

ADENOVIRUSES

Adenoviruses are non-enveloped double-stranded DNA viruses of 60–90 nm diameter. They can cause relatively mild, self-limiting diseases of the upper respiratory tract ("colds"), gastroenteritis, or conjunctivitis, but most infections are asymptomatic. Importantly, adenoviruses have not been associated with any neoplastic disease in humans (Benihound *et al.*, 1999). The natural targets for adenoviruses are epithelial cells of the respiratory and gastrointestinal tracts. Following systemic intravenous injection, however, the viruses are preferentially cleared by the liver, which leads to highly efficient infection of both hepatocytes and liver endothelial cells (Chan, 1995).

Uptake of the adenovirus particle is a two stage process involving an initial interaction of the fiber protein with a range of cellular receptors,

which include the coxsackievirus-adenovirus receptors (CAR) and major histocompatibility complex (MHC) class I (Bergelson *et al.*, 1997). Then, the penton base protein binds to the α_v -containing integrins allowing internalization *via* receptor-mediated endocytosis (Wickham *et al.*, 1993). The toxic activity of pentons is also responsible for the rupture of the phagocytic membrane and release of the virus into the cytoplasm. Partially uncoated particles migrate to the nucleus, where the DNA enters through nuclear pores, whereupon it is converted into a virus DNA-cell histone complex. Viral DNA does not integrate into cell chromosomes (Mountain, 2000).

After infection with adenoviruses, an immune response rapidly develops. First, neutrophil and macrophage infiltration occurs and presentation of exogenous capsid antigens by MHC-II molecules activates T helper (Th) lymphocytes of both

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Abbreviations: AAV, adeno-associated viruses; apo, apolipoprotein; CTL, cytotoxic T lymphocyte; DMD, Duchenne muscular dystrophy; HdAd, helper-dependent adenoviral vectors; ITR, inverted terminal repeat.

Th1 and Th2 phenotype, driving the cytotoxic and humoral responses, respectively. Then, MHC-I-restricted, cytotoxic T lymphocytes (CTL) trigger the specific immunoclearance of the infected cells (Kovesdi *et al.*, 1997; Benihound *et al.*, 1999). As a consequence of infection, low levels of IgG immunoglobulins against adenoviral antigens are sustained in the blood, which dramatically enhances the response against virus particles during the next infection (Kafri *et al.*, 1998). Of importance, the majority of the human population will have experienced at least one infection by age 10 and about 80% of adults have preexisting antibodies against adenoviruses (Eto *et al.*, 2004).

ADENOVIRAL VECTORS OF THE FIRST GENERATION

The genome of adenoviruses consists of 36 kb of double-stranded linear DNA with inverted terminal repeat (ITR) sequences at each end. Expression of genes occurs in two phases, early and late, defined by the onset of DNA replication (Benihound *et al.*, 1999). Early genes are encoded by four distinct transcription units, E1–E4, whereas the late gene products are generated from a single promoter. Importantly, proteins encoded by E1 region are indispensable for further viral gene expression and DNA replication (Wang & Huang, 2000).

Vector of the first generation is prepared by substitution of a transgene in the place of E1 (or E1 and E3) genes (Fig. 1). The resulting construct has a cloning capacity up to 9 kb. An E1-deficient (Δ E1) vector is replication-defective and must be propagated in a permissive cell line, engineered to provide E1 functions in *trans* (Chan, 1995; Gerard & Chan, 1996). Substitution for the E3 genes is not necessary, as they are involved in inhibition of host immune antiviral response, not in the replication of the adenoviral genome. Among the 51 human adenovirus

serotypes discovered to date, Ad5 and Ad2 are most commonly used for generation of vectors (Wang & Huang, 2000).

Adenoviral vectors give the most efficient gene transfer in comparison with other systems (Mountain, 2000). Moreover, they can transduce both dividing and post-mitotic cells of nearly all human tissues – including skin, muscle, blood vessel, bone, nerve, and liver (Benihound *et al.*, 1999; Mountain, 2000). They can be also effective in delivering genes into some leukocytes (Buttgereit *et al.*, 2000). Following delivery, transgene expression is at a very high level. Unfortunately, it decreases rapidly after several days, being low or undetectable after several weeks (Mountain, 2000).

Many studies have clearly demonstrated that adenoviral vectors of first generation induce innate and adaptive immune responses *in vivo* against viral capsids and transduced cells (Wilmott *et al.*, 1996; Muruve *et al.*, 2004). Despite the deficiency in the E1 region, the vectors are, in fact, not completely replication-defective (Zhang *et al.*, 1998). They deliver the transgene along with the residual viral genes and indeed synthesize some viral proteins (Dai *et al.*, 1995). Even low level expression of these leads to activation of CTL and results in destruction of expressing cells, being the major cause of transgene silencing (Mountain, 2000).

HELPER-DEPENDENT ADENOVIRUS VECTORS (HdAd)

Helper-dependent adenoviral vector (HdAd), called also “gutless” or “guttled” vector, is created by removing all viral genes and leaving only the ITRs necessary for vector propagation, and the Ψ sequence required for packaging (Fig. 1) (Mitani *et al.*, 1995; Kochanek *et al.*, 1996; Parks *et al.*, 1996). Any unused space is occupied by a transgene or by non-coding “stuffer” DNA sequence, to build the vector to a size suitable for packaging (Hardy *et al.*, 1997) (Fig. 1). This strategy completely eliminates the production of viral proteins in infected cells. Therefore, HdAd elicits only minute or negligible CTL response and is capable of producing long-term gene expression (Morral *et al.*, 1998; Morsy *et al.*, 1998a). Additional advantage of HdAd is a high cloning capacity, extended up to 37 kb (Mitani *et al.*, 1995).

The nature of the DNA backbone can have important effects on the functioning of the HdAd virus, which must be taken into account in the design of vectors. The most commonly used stuffer DNA originates from human hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Parks *et al.*, 1999).

The first step in generation of HdAd constructs is based on ligation of the expression cassette into large plasmids containing the viral ITRs flank-

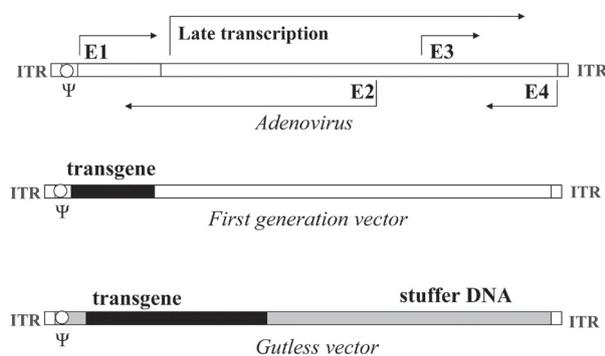


Figure 1. Scheme of the adenovirus, first generation adenovirus and gutless adenovirus genomes.

Viral sequences are depicted in white. ITR, inverted terminal repeats, necessary for genome propagation; Ψ , packaging sequence. After Józkowicz *et al.* (2002), modified.

ing stuffer DNA. This process, although commonly applied, is generally very inefficient. It has been shown, however, that similarly as in the case of the $\Delta E1$ adenoviral vectors, plasmids for generation of HdAd constructs can be obtained by homologous recombination in *Escherichia coli*, which potentially can facilitate the production (Toietta *et al.*, 2002).

A number of systems have been also developed for rescuing HdAd. The most common method for propagation and purification of HdAd utilizes two elements: i) a helper virus ($\Delta E1/\Delta E3$) with Ψ sequence flanked by loxP sites (Fig. 2) and ii) a modified 293cre cell line expressing Cre recombinase which catalyzes recombination between loxP sequences, excising the packaging site from helper DNA. Such a deletion renders the helper genome unpackageable but leaves all other viral functions intact, retaining the ability to replicate and provide helper functions (Fig. 3) (Parks *et al.*, 1996; Hardy *et al.*, 1997). The efficacy of this technology is sufficient for experimental gene therapy, but is not suitable for production and purification of HdAd vectors at a scale necessary for clinical use (Sakhuja *et al.*, 2003). To increase the efficiency of the system, both improved helper viruses and alternative gutless vector producer cell lines are necessary. One of the promising modifications is establishing the cells adapted to serum-free suspension culture, which can allow obtaining high-titer gutless vector preparations by using bioreactor technology (Sakhuja *et al.*, 2003; Palmer & Ng, 2005).

An additional problem to be solved is contamination with helper $\Delta E1$ virus, which may have substantial unwanted effects. Unfortunately, a helper virus, even lacking a packaging signal, still can be packaged, although at a low frequency (about 0.1%) (Sakhuja *et al.*, 2003). It seems that this level of impurity cannot be further decreased using the Cre/loxP system, as Cre recombinase in 293cre cells per-

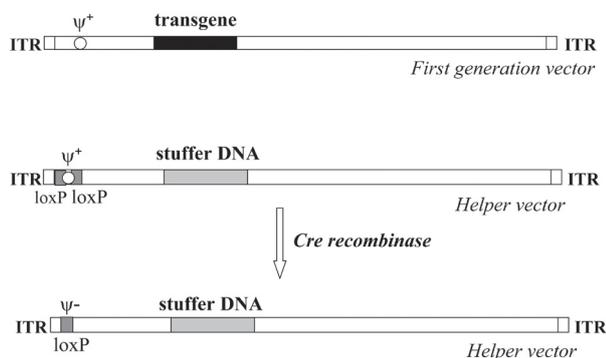


Figure 2. Schematic comparison of the first generation adenoviral vector and helper vector.

Note that in the helper genome the transgene is replaced by stuffer DNA, while the packaging sequence Ψ is flanked by loxP sites. In the presence of recombinase cre, Ψ is excised from the helper genome.

mits some helper viruses to escape packaging signal excision and propagate (Ng *et al.*, 2002). Thus, the resulting viral stocks must be additionally purified by ultracentrifugation in CsCl, which can produce HdAd stocks with very low (less than 0.01%) helper contamination (Hardy *et al.*, 1997).

This separation step hinders, however, large-scale production of clinical-grade HdAd virus. One of the possible solutions is using a more efficient recombinase, e.g. modified FLP (FLPe), to remove Ψ from the helper virus in the producer cells. FLP has been shown to mediate maximum levels of excision close to 100% compared to 80% for Cre. Thus, it is postulated that the FLPe-based system may yield HdAd with very low levels of helper virus contamination without the need for ultracentrifugation, which should allow large-scale generation of gutless vectors by means of column chromatography (Umana *et al.*, 2001). Finally, the safety of the HdAd vectors can be further optimized by using double-deleted $\Delta E1\Delta E2a$ helper virus (Zhou *et al.*, 2001).

Gutless adenoviruses can infect a wide range of mammalian tissues, independent of the replicative state of the cells. Like $\Delta E1$ particles, intravenously injected HdAd vectors transport the transgene preferentially to the liver, where infection efficacy may reach almost 100%. After local injection, however, they can ensure efficient expression in other organs as well (Bramson *et al.*, 2004; Palmer & Ng, 2005). Nevertheless, certain serotypes of adenoviruses have diverse tissue tropisms *in vivo*, and in some tissues, e.g. in skeletal muscles, the efficacy of adenoviral vectors is relatively low. Particularly resistant are some lymphoid cells (Bramson *et al.*, 2004). This dis-

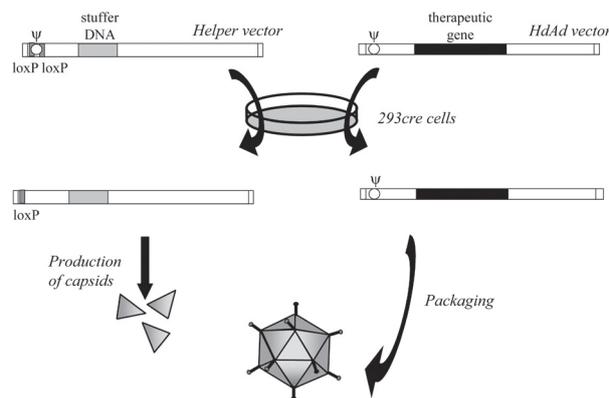


Figure 3. Production of helper-dependent adenoviruses using cre-loxP system.

After cotransfection of 293cre cells with genomes of the helper vector and helper-dependent vector, viral proteins necessary for genome replication and capsid formation are produced by helper virus. However, in the presence of recombinase cre the Ψ sequence, flanked by loxP sites, is removed from the helper genome. Therefore, only HdAd DNA can be packaged into viral capsids. After Parks *et al.* (1996), simplified.

advantage can be overcome through modification of adenovirus fiber composition. For example, a chimeric Ad5/Ad35 fiber incorporated into an HdAd construct augmented the infection of K562 lymphoid cells approx. 50 times (Balamotis *et al.*, 2004), whereas incorporation of a polylysine motif resulted in a 60–70% increase in transduction efficiency in adult skeletal muscles (Bramson *et al.*, 2004).

In the liver, expression of the transgene is limited to several weeks with Δ E1 Ad vectors (Morrall *et al.*, 1999). In contrast, single delivery using HdAd may result in a strong expression lasting for longer than a year or even 2.5 years, as shown in baboons and mice, respectively (Morrall *et al.*, 1999; Kim *et al.*, 2001). Furthermore, some studies have demonstrated the feasibility of circumventing the humoral response to the virus by sequential injection with vectors of different serotypes. These observations suggest that very long-term expression should be possible by re-administration of the transgene using different HdAds (Morrall *et al.*, 1999; Kim *et al.*, 2001).

It has been postulated that recombinant adenoviral vectors have some mechanism ensuring their persistence during cell cycling. When rapid hepatocyte cell divisions in mouse liver were induced by partial hepatectomy, a 55% reduction in transgene expression from an HdAd vector was observed after one month. In contrast, in plasmid DNA-injected mice it decreased by 95% (Ehrhardt *et al.*, 2003). Nevertheless, expression of a transgene delivered by either Δ E1 Ad or HdAd vectors is transient in proliferating cells, since such vectors do not possess elements that allow replication and segregation of the replicated genomes to daughter cells (Kreppel & Kochanek, 2004).

In contrast to adenoviral systems, Epstein-Barr virus (EBV) episomes are stably maintained in permissive proliferating cell lines due to EBV nuclear antigen 1 (EBNA-1)-mediated replication and segregation. Thus, to achieve sustained transgene expression in proliferating cells, a binary HdAd-EBV hybrid system has been developed that consists of one HdAd vector for the expression of recombinase and a second HdAd vector that harbors all of the sequences for the EBV episome, containing the transgene and EBNA-1 expression cassettes, flanked by recombinase-recognized sites. Upon coinfection of cells, the EBV episomes are circularized by recombinase expressed from the first vector. Such hybrid viruses have about 40% infection efficacy *in vitro* (Dorigo *et al.*, 2004). Depending on the system, up to 98% of the circularized genomes are replicated and segregated to daughter cells, and transgene expression in proliferating cells can be observed for over 20 weeks (Dorigo *et al.*, 2004; Kreppel & Kochanek, 2004). This novel gene transfer system may have the potential to confer long-term episomal transgene expression and therefore to correct genetic defects with

reduced vector-related toxicity and without the risk of insertional mutagenesis (Dorigo *et al.*, 2004).

Furthermore, adenoviral vectors randomly integrate into host chromosomes at frequencies of 0.001–1% of infected cells, and gene expression from the integrated vector persists for at least 50 cell divisions (Harui *et al.*, 1999; Mitani & Kubo, 2002). Of note is that even extensive homologies and abundant chromosomal repeat elements present in the vector backbone did not lead to integration of the transgene expression cassette *via* homologous or homology-mediated mechanisms (Hillgenberg *et al.*, 2001).

To improve the integration frequencies, a variety of hybrid vectors combining the highly efficient DNA delivery of HdAd adenovirus with the integrating machinery of transposons, retroviruses, and adeno-associated viruses (AAV) have been emerging. These hybrid vectors have shown promise, at least in *in vitro* systems (Mitani & Kubo, 2002). For example an HdAd-transposon hybrid vector has proven useful in sustaining stable expression of a transgene through integration into host cell chromosomes. Systemic *in vivo* delivery of this system, which utilizes a donor Sleeping Beauty (SB) transposon vector that undergoes FLP-mediated recombination, resulted in efficient generation of transposon circles and stable SB transposase-mediated chromosomal integration in mouse liver. Somatic integration was sufficient to maintain therapeutic levels of human coagulation Factor IX for more than six months in mice undergoing extensive liver proliferation (Yant *et al.*, 2002).

Similarly, a chimeric system combining HdAd vector and L1 retrotransposon has been elaborated. Efficient adenovirus-mediated delivery of the L1 element into cultured human cells resulted in subsequent retrotransposition and stable integration of the transgene (Soifer *et al.*, 2001; Soifer & Kasahara, 2004). Another possibility is employing an HdAd virus as a carrier to deliver a genome of fully functional retroviral vector. This experimental system can transduce the cells with permanent integration and progressive spread of transgene expression (Soifer *et al.*, 2002).

Finally, HdAd viruses can be used to deliver an expression cassette together with the Rep68/78 gene or AAV-ITRs, two elements which are necessary for integration into an AAV specific locus of human chromosome 19 (AAVS1). Despite the low efficacy of vector rescue, DNA integration at the AAVS1 site was demonstrated in hepatoma cells coinfecting with HdAd expressing Rep78 and with the second HdAd vector carrying a transgene flanked by AAV-ITRs. The high transduction efficiency, large cloning capacity, and high titer of HdAd, combined with the site-specific integration machinery provided by the AAV-derived components, can make the Ad/AAV hybrid viruses a promising vehicle for gene therapy (Recchia *et al.*, 1999).

IMMUNOLOGICAL RESPONSE TO ADENOVIRAL VECTORS

Gutless adenoviruses display strongly reduced toxicity and immunogenicity compared to early generation vectors (Sakhujia *et al.*, 2003). Especially, a CTL-mediated response, very strong in the case of $\Delta E1$ carriers, is usually negligible (Morral *et al.*, 1998; Morsy *et al.*, 1998a). It must be stressed, however, that high doses of $\Delta E1$ viruses cause long-term hepatotoxicity not only due to viral gene expression from the vector backbone, but also an acute innate inflammatory reaction in response to the capsid proteins (Brunetti-Pierri *et al.*, 2004).

Intravascular injection of adenoviral vectors may result in a toxic and potentially lethal reaction, the mechanism of which is poorly understood (Schiedner *et al.*, 2003). It can induce early expression of inflammatory cytokine and chemokine genes in the liver, including interferon-inducible protein-10 (IP-10), macrophage inflammatory protein-2 (MIP-2), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF α), leading to the recruitment of CD11b-positive leukocytes to the transduced liver within hours of administration (Muruve *et al.*, 2004; Mok *et al.*, 2005). Furthermore, systemic delivery of adenoviral vectors in mice may lead within minutes to a transient change in behavior that is characterized by inactivity and lethargy. Accordingly, immediately after injection a significant drop in blood pressure can be measured probably due to the systemic activation of endothelial cells and increased production of nitric oxide (NO), resulting from activation (followed by destruction) of Kupffer cells (Schiedner *et al.*, 2003). This acute toxicity is neither prevented nor reduced for HdAd, as the capsid composition and structure remains unchanged (Stilwell *et al.*, 2003; Brunetti-Pierri *et al.*, 2004). Recent data have demonstrated, however, that polyethylene glycol (PEG) modification of adenovirus can protect HdAd vectors from preexisting immune responses by reducing protein-protein interactions. The reduced innate reaction paralleled similar reductions in vector uptake by Kupffer cells (Mok *et al.*, 2005).

COMPARISON OF HdAd AND $\Delta E1$ ADENOVIRUSES

HdAd vehicles have been tested in several animal models, with very promising results. The most convincing evidence of superiority of HdAd vectors over other types of adenoviral vehicles is supplied by a direct comparison of therapeutic benefits resulting from delivering the same gene using different vector systems. For example, in experiments performed in mice, a systemic intravenous injection of erythropoietin gene using HdAd vector

produced a 100-fold higher expression per infectious particle when compared to a first generation virus. The expression was long-lasting and resulted in a significant increase in hematocrit for more than six months (Maione *et al.*, 2000). Also comparing to a $\Delta E1/\Delta E2a/\Delta E3$ vector, the gutless adenovirus produced ten-fold higher and much longer expression of factor VIII (FVIII) in hemophilia-A mice (Reddy *et al.*, 2002). Finally, a study on the duration of expression of the α_1 -antitrypsin (hAAT) gene delivered to the liver of baboons has demonstrated that administration of HdAd resulted in transgene expression for longer than a year in comparison to three months obtained from first generation vehicles (Morral *et al.*, 1999).

Similar differences were also observed when HdAd and $\Delta E1$ vectors, both coding for urokinase plasminogen activator (uPA), were delivered locally into the carotid artery in rabbits. The expression of uPA was lost, and vector DNA declined rapidly in arteries infused with first generation vehicles, whereas it persisted for at least two months in HdAd-treated vessels. Moreover, these arteries had significantly less inflammation and neointimal formation than the vessels treated with the $\Delta E1$ adenoviruses (Wen *et al.*, 2004). Considerably weaker inflammation was also found in the rat myocardium injected with HdAd, as illustrated by less abundant infiltrates of macrophages or lymphocytes and by reduced levels of pro-inflammatory cytokines. Thus, gutless vectors may be superior to earlier-generation adenovirus vectors for cardiovascular gene therapy (Fleury *et al.*, 2004).

LOCAL INJECTIONS OF HdAd VECTORS

After local injection, gutless vectors can provide efficient expression in many tissues, e.g. in skeletal muscles. It has been found that low doses of HdAd can direct long-lasting gene expression in the muscles of mice, with 100% of treated animals showing sustained expression after 4 months. Moreover, this route of gene delivery can be also used in the presence of a preexisting immunity to adenovirus. This suggests that gene therapy by intramuscular delivery of HdAd vectors is feasible (Maione *et al.*, 2001).

The toxicity and immunogenicity of first generation adenoviral vectors prohibited the application of gene transfer in the majority of central nervous system disorders. Zou and colleagues (2000; 2001) compared the effect of injection of $\Delta E1$ and HdAd adenoviral vectors into the brain of adult rats. They found that first generation vector-mediated gene expression decreased rapidly, becoming undetectable after 1–2 months, while expression from HdAd persisted for more than half a year. In addition, in

contrast to the effects of $\Delta E1$ vectors, secretion of proinflammatory cytokines was minimal after HdAd vector injection, resulting in reduced numbers of activated microglial cells, astrocytes, and infiltrating macrophages in brain tissue. These findings indicate that HdAd vehicles should provide a safe and effective means to transfer therapeutic genes into the brain (Zou *et al.*, 2000; 2001).

Notably, recent study has shown that helper-dependent canine adenovirus (CAV-2) vectors can preferentially transduce neurons and efficiently travel *via* axonal retrograde transport. One year after striatal injections into the rat brain, a stable high level of transgene expression was found in neurons of the striatum, substantia nigra, and in the basal nuclei of Meynert. This suggests that helper-dependent CAV-2 vectors may be clinically relevant for the treatment of many neurodegenerative diseases (Soudais *et al.*, 2004).

SPECIFIC OR REGULABLE PROMOTERS

The ability to target specific tissues is important in many applications of gene therapy. In this respect, a disadvantage of adenoviral vectors is the relative lack of specificity with which they transduce cells. One approach to overcome this is to express the therapeutic gene under the control of a tissue-specific promoter. However, the specificity and activity of such promoters may be altered by the adenoviral sequences in the vector backbone (Shi *et al.*, 2002). In contrast to the first generation adenoviruses, HdAd does not contain any *cis*-acting viral sequences which could interfere with the specificity of the inserted promoters (Shi *et al.*, 2004). Accordingly, direct comparison has evidenced that ligand-inducible transcriptional regulation systems act much better in the context of gutless adenoviruses than in the vectors of earlier generations (Shi *et al.*, 2002; Zerby *et al.*, 2003).

Gutless vectors are also suitable to delivering a transgene regulated by ligand-inducible sequences. For example, in mice injected with an HdAd vector containing the interferon- α (IFN α) gene under control of a tetracycline inducible transactivator, high levels of serum IFN α appeared only after transcriptional activation. Furthermore, it was efficiently reinducible to the same high level up to 3 months post injection, and the amount of expressed cytokine could be regulated by dosing doxycycline. Of importance, such a treatment resulted in prolonged survival and reduced liver damage in mice challenged with a lethal dose of coronavirus (Aurisicchio *et al.*, 2001).

Similarly, systemic injection of HdAd viruses containing an RU486-inducible system for liver-specific generation of interleukin-12 (IL-12) resulted in

expression of the transgene only in the liver of mice, despite the presence of the vector in many different tissues. Furthermore, the RU486-induced synthesis of IL-12 caused a complete regression of liver metastases in all animals treated. Thus, gutless adenoviral vectors allowing liver-specific and regulable transgene expression for prolonged periods of time can be promising tools for gene therapy of liver cancer and could also be useful for other forms of hepatic diseases (Wang *et al.*, 2004).

Finally, some promoters can be used to drive the transgene expression specifically in tumor cells. It has been shown, for instance, that the promoter of secretoglobulin family 2A member 2 (SCGB2A2, also known as mammaglobin-1) is highly and preferentially active in breast cancer cells when introduced *via* an HdAd vector. This system could be, perhaps, particularly advantageous for transfer of toxic genes to mammary tumors (Shi *et al.*, 2004).

HdAd IN TREATMENT OF DISEASES IN ANIMAL MODELS

Currently HdAd vectors are being tested in experiments determining the potency of gene therapy strategies in preventing and treatment of inherited diseases in animal models.

Several experiments focused on hemophilia caused by deficiency of Factor VIII (FVIII) or Factor IX (FIX). Many different approaches *via* gene transfer have been attempted in animal models but all have potential drawbacks (Ehrhardt *et al.*, 2003). Methods based on $\Delta E1$ adenoviruses have been ineffective primarily due to the immunogenicity of these vectors (Brown *et al.*, 2004b). On the contrary, HdAd vectors have produced hemostatic correction in animal models. In a dog with hemophilia-B, FIX plasma concentrations were sustained for up to 2.5 months at a therapeutic range and normalization of whole blood clotting time for about a month. This was followed by a decrease and stabilized partial correction for 4.5 months. In sharp contrast to other studies using earlier generations of adenoviral vectors, there was no vector-related elevation of liver enzymes, no fall in platelet counts, and liver histology was normal (Ehrhardt *et al.*, 2003).

Similarly, in hemophilic mice treated with HdAd vectors harboring FIX or FVIII, high and sustained levels of therapeutic proteins were obtained in the serum, without or with minimal unwanted side-effects (Ehrhardt & Kay, 2002; Reddy *et al.*, 2002; Brown *et al.*, 2004b). However, the first patient suffering from severe hemophilia-A to be treated with a gutless adenoviral vector expressing full-length FVIII, and who appeared to have an FVIII level one-hundredth of the normal one for several months, developed a transient inflammatory response with

hematologic and liver abnormalities (Chuah *et al.*, 2004).

Several studies have also been devoted to the treatment of Duchenne muscular dystrophy (DMD), a lethal degenerative disease resulting from the absence of the dystrophin protein at the muscle membrane (Haecker *et al.*, 1996; Kochanek *et al.*, 1996).

Full-length dystrophin cDNA is too long to be accommodated in an adenoviral vector of the first generation. It can be easily introduced, however, to an HdAd vehicle. Experiments performed in mdx mice (animal model for DMD) evidenced high, local expression of dystrophin cDNA after intramuscular injection of an HdAd construct. The activity of the transgene was accompanied by restoration of dystrophin-associated cytoskeletal proteins and histological improvement of dystrophic muscle (Haecker *et al.*, 1996; Kochanek *et al.*, 1996; Dudley *et al.*, 2004). Unfortunately, transgene expression was usually short-lasting (Kochanek *et al.*, 1996). Possibly, the immune response induced by transgene protein was responsible for the loss of vector activity (Chen *et al.*, 1997). This observation illustrates the general problem of supplementary gene therapy — where the transgene product (e.g., normal dystrophin protein), when introduced to an animal or patient deficient in the wild type gene, can be recognized as a potential target for immune responses (Haecker *et al.*, 1996).

Interestingly, the success of therapy and the strength of immunological response may depend on the age of treated animals, as demonstrated in an elegant study where muscles of neonatal and juvenile mdx mice were injected with an HdAd vector encoding two full-length murine dystrophin cDNAs. At 10 days post-injection of neonatal muscles, 42% of the total number of fibers were dystrophin-positive, a value that did not decrease for at least 6 months. In treated juveniles, maximal transduction was lower (24% of fibers), and it further decreased during 6 months. All the aspects studied of the physiology including maximal force-generating capacity and resistance to contraction-induced injuries were significantly improved in neonatally treated mice, whereas the therapeutic effects in the older animals were much worse. In both groups a strong humoral response against murine dystrophin was observed but a mild inflammatory response occurred only in the juveniles (Gilbert *et al.*, 2003).

Local delivery of HdAd vectors may be also used to regulate angiogenesis, the formation of new blood vessels from preexisting capillaries. For example, subretinal injection of a gutless adenovirus containing an expression construct for angiopoietin-2 (Ang2) was able to perturb retinal pigmented epithelial (RPE) cells and, together with overexpression of vascular endothelial growth factor (VEGF), induce choroidal neovascularization (Oshima *et al.*, 2004).

Accordingly, subretinal injection of an HdAd vehicle designed to provide expression of endostatin, an endogenous inhibitor of angiogenesis, significantly decreased vascular leakage induced by overexpression of VEGF. Thus, one can suppose that in patients with diabetic retinopathy, endostatin gene transfer by a gutless vector may provide a way to decrease the risk of the major causes of visual loss: macular edema, neovascularization, and retinal detachment (Takahashi *et al.*, 2003).

Promising results have also been obtained with correction of leptin disorders. Leptin is a potent modulator of food intake and its deficiency leads to uncontrolled appetite, obesity, and increased blood insulin and glucose levels (Campfield *et al.*, 1995). Morsy and colleagues (1998b) compared the safety and efficacy of leptin delivery mediated by HdAd and a first generation vector in leptin-deficient mice. In contrast to a $\Delta E1$ virus, HdAd leptin delivery did not induce any liver toxicity, inflammation or cellular infiltrates, and resulted in prolonged elevation of serum leptin levels, leading to significant weight loss in the treated animals.

Gene therapy was also proposed as a potential approach for treatment of one of the most common urea cycle disorder, ornithine transcarbamylase (OTC) deficiency (Raper *et al.*, 1998). Unfortunately, in the single human clinical trial using $\Delta E1$ adenovirus coding for the OTC gene one patient died after delivery of a high dose of the vector. Moreover, this trial did not show any biochemical correction in the 18 patients enrolled (Raper *et al.*, 2003). In accordance, in OTC-deficient mice treated with $\Delta E1/\Delta E2$ adenovirus expressing the OTC gene, the therapeutic effect was only transient (Ye *et al.*, 1997). In contrast, a recent paper describes that HdAd vector was able to produce metabolic correction in mice for >6 months. This treatment resulted in normalized orotic aciduria, normal hepatic enzyme activity, and elevated OTC mRNA and protein levels in the absence of chronic hepatotoxicity. This may suggest potential usefulness of HdAd vehicles for treating human in-born errors of hepatocyte metabolism, like the urea cycle disorders, that require high-level transduction for clinical correction (Mian *et al.*, 2004).

Since HdAd vectors have a natural tropism to the liver, they can be a very promising tool for gene therapies in inherited lipid metabolism disorders. Liver is a central organ in lipid regulation and transfer of a single gene involved in lipoprotein turnover could effectively interact with other key regulatory molecules (Gerard & Collen, 1997).

One of the potential targets for HdAd vector-mediated gene therapy is familial hypercholesterolemia (FH), a disease resulting from low density lipoprotein receptor (LDLR) deficiency, which affects approx. 1 in 500 persons in the heterozygous state and 1 in one million persons in the homozygous

state (St. Clair & Beisiegel, 1997). A model of human FH are low density lipoprotein receptor (LDLR)-deficient mice. It has been shown that a low-dose HdAd vector harboring the LDLR gene efficiently protects LDLR^{-/-} mice from development of atherosclerosis for at least 60 weeks. At the same time, in control animals atherosclerosis lesions covered approx. 50% of the surface of aortas. Moreover, the lipid-lowering effect of HdAd-LDLR lasted for at least 108 weeks (>2 years), by which time all control mice had died. In addition to retarding lesion progression, the treatment caused lesion remodeling from a vulnerable-looking to a more stable-appearing phenotype (Nomura *et al.*, 2004).

Another approach to correcting hypercholesterolemia in LDLR^{-/-} mice employed a gutless vector encoding very light density lipoprotein receptor (VLDLR) (Oka *et al.*, 2001). VLDLR binds apolipoprotein E (apoE) and can mediate the uptake of proatherogenic particles like chylomicrons and VLDL remnants, intermediate density lipoprotein (IDL) or lipoprotein-a (Lp(a)). VLDLR is present in many cell types, e.g. in skeletal muscle fibres, adipocytes, macrophages or foam cells, but it is not expressed in the liver (St. Clair & Beisiegel, 1997). The superiority of VLDLR over the "natural" receptor (LDLR) was attributable to the fact that LDLR could be recognized as a neoantigen by LDLR-deficient mice, which mounted humoral and cellular immune response to the LDLR, leading to the extinction of transgene expression. Ectopic liver expression of VLDLR did not induced any immune response (Kobayashi *et al.*, 1996; Nomura *et al.*, 2004). In effect, VLDLR gene therapy in LDLR-deficient mice produced a substantially more sustained hypocholesterolemic response than did LDLR: the lowering of plasma cholesterol lasted for at least 6 months and was associated with almost complete prevention of atherosclerosis (Oka *et al.*, 2001).

Similar anti-atherosclerotic effects were also obtained after injection of LDLR^{-/-} or apoE^{-/-} mice with an HdAd vector encoding apolipoprotein-AI (apoAI), the major protein component of high-density lipoproteins (HDL) (Belalcazar *et al.*, 2003). Overexpression of apoAI increased HDL cholesterol and decreased atherosclerosis in the absence of detectable hepatic toxicity (Pastore *et al.*, 2004).

The most spectacular outcome was achieved by Chan and coworkers (including one of us, A.J.), using a gutless adenovirus harboring apoE gene. ApoE-deficient mice, which display spontaneous hypercholesterolemia, were treated with a single injection of an HdAd vector expressing apoE at 12 weeks of age. This treatment normalized plasma apoE concentration and produced an immediate fall in plasma cholesterol to the values typical for wild-type mice, without any side-effects. Importantly, the correction lasted for at least 2.5 years, the natural

life-span of the mice, and resulted in complete protection against atherosclerosis (Kim *et al.*, 2001).

Finally, Chan and coworkers have demonstrated that HdAd can be used for the treatment of diabetes. Administration of a mixture of vectors coding for betacellulin and a pancreatic islet transcription factor, Neurod, into streptozotocin-treated mice, induced islet neogenesis in the liver, which completely reversed diabetes and normalized glucose level for at least four months (Kojima *et al.*, 2003).

CONCLUSIONS

HdAd vectors give rise to long-term sustained transgene expression in both small laboratory animals and nonhuman primates. They are as efficient as the earlier generation adenoviral vehicles, but substantially less toxic. Thus, they appear to be one of the most promising tools for gene therapy, especially the therapy directed to the liver, skeletal muscle, brain, or cardiovascular system. The large insert capacity of the gutless vector allows for simultaneous expression of several genes, introduction of long cDNA (e.g., dystrophin) or genomic DNA, and addition of the long regulatory DNA regions preserving their cell-type specificities.

There are, however, some important pitfalls. Although gutless adenoviruses do not induce T lymphocyte-mediated cytotoxicity, their capsid, crucial for the efficient transgene delivery, contains all of the viral proteins being a potential target for neutralizing antibodies. Acute toxicity provoked by the capsid, especially after systemic, intravascular injection, is the most significant obstacle currently hindering clinical application. This immune response specific for the antigenic viral capsid proteins could not be alleviated to date. It seems, however, that local delivery of the vector or administration of PEGylated capsids can possibly allow one to bypass a preexisting humoral immunity and attenuate the acute toxicity. Thus, HdAd vectors, if used with caution, may have a great potential for efficient gene delivery both in experimental gene therapy and in clinical trials.

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