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REVIEW

Gene therapy: light is finally in the tunnel

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ABSTRACT

After two decades of ups and downs, gene therapy has recently achieved a milestone in treating patients with Leber's congenital amaurosis (LCA). LCA is a group of inherited blinding diseases with retinal degeneration and severe vision loss in early infancy. Mutations in several genes, including *RPE65*, cause the disease. Using adeno-associated virus as a vector, three independent teams of investigators have recently shown that *RPE65* can be delivered to retinal pigment epithelial cells of LCA patients by subretinal injections resulting in clinical benefits without side effects. However, considering the whole field of gene therapy, there are still major obstacles to clinical applications for other diseases. These obstacles include innate and immune barriers to vector delivery, toxicity of vectors and the lack of sustained therapeutic gene expression. Therefore, new strategies are needed to overcome these hurdles for achieving safe and effective gene therapy. In this article, we shall review the major advancements over the past two decades and, using lung gene therapy as an example, discuss the current obstacles and possible solutions to provide a roadmap for future gene therapy research.

KEYWORDS gene therapy, transgenes, viral vector, non-viral vector, helper-dependent adenoviral vector, adeno-associated virus, lentivirus, cystic fibrosis transmembrane conductance regulator (CFTR), host immune responses

INTRODUCTION

The potential of gene therapy in medical applications was recognized soon after the discovery of DNA as genetic material and of genetic information flow (from DNA to RNA to protein). The early history of gene therapy was extensively reviewed by Wolff and Lederberg (Wolff and Lederberg, 1994) and we shall only highlight a few points here prior to the discussion of vectors and approaches used in gene therapy. Attempts at human gene therapy were initiated in the late 1960s and early 1970s when S. Rogers injected the Shope papilloma virus into patients with arginase deficiency, based on his initial observation that the virus induced high levels of arginase activity in rabbit skin tumors and might contain an arginase gene (Rogers and Moore, 1963). Although the attempt eventually turned out to be unsuccessful (Terheggen et al., 1975), it demonstrated the early enthusiasm in gene therapy. Due to the lack of basic understanding of gene expression and effective methods for gene delivery, the lack of success in early attempts at gene therapy is now understandable.

In the past two decades, more than 1500 gene therapy clinical trials have been conducted with various viral and nonviral vectors for diseases such as lung diseases (e.g. cystic fibrosis), immunodeficiencies and eye diseases (Koehler et al., 2001b; St George, 2003; Flotte et al., 2007; Aiuti et al., 2009; Herzog et al., 2010; Stein et al., 2011). However, in most cases, therapeutic efficiency was not satisfactory or adverse effects became major concerns. Over the past few years, major research efforts in gene therapy were directed toward developing safe and efficient gene therapy reagents and understanding the problems associated with gene delivery, such as acute toxicity associated with adenoviral vectors (Morral et al., 2002) and insertional mutagenesis with lentiviral vectors (Aiuti et al., 2009; Herzog et al., 2010; Stein et al., 2011), further improving gene therapy vectors (Parks, 2000; Koehler et al., 2001a; Barquinero et al., 2004; Ferrari et al., 2004; Ferrari et al., 2007) and exploring experimental conditions for enhancing the efficiency of gene delivery (Limberis et al., 2002; Flotte et al., 2007; Kushwah et al., 2007). In this review, we shall first briefly describe vectors

commonly used for gene delivery to animal models and humans, followed by discussing current problems and possible solutions associated with gene therapy.

VECTORS FOR GENE THERAPY

There are two general approaches being employed in gene therapy based on vectors used for gene delivery. One approach employs recombinant viruses to deliver genes and the other uses non-viral vectors. In general, transfer of therapeutic genes is most efficient when delivered by viral vectors, but the major obstacle to the use of such viral vectors is their immunogenicity. Among the viral vectors developed for *in vivo* gene delivery, we shall briefly describe the vectors derived from adenovirus (Ad), adeno-associated virus (AAV), and retrovirus since these vectors have been used in clinical trials. Progress has also been made in vectors derived from other viruses, such as herpes simplex virus (Glorioso et al., 1994) and Sendai virus (the murine parainfluenza virus type 1) (Ferrari et al., 2004, 2007). Due to the space limitation, however, these vectors will not be covered in this review. Among the non-viral vectors, only cationic liposomes were have been extensively explored and thus, will be discussed.

Viral vectors

Adenoviral vectors

Adenoviruses are a family of DNA viruses with a linear double-stranded genome. Their viral coat proteins are organized in an icosahedral, non-enveloped capsid with fiber-like projections from each of the 12 vertices (Kojaoghlanian et al., 2003). In addition to the fiber, the other two major types of capsid proteins are the hexon that forms each geometric face of the capsid and the penton base that anchors the fiber. There are at least 51 human Ad serotypes that are classified into six subgroups (A–F) according to various properties (Kojaoghlanian et al., 2003). Members in subgroup C, such as Ad2 and Ad5, are non-oncogenic and predominantly used as vectors for gene delivery (Cao et al., 2004). The viral genome, which is about 36 kb, is experimentally divided into early and late regions based on whether genes in a region are expressed before or after DNA replication. Early regions, E1a and E1b, contain genes encoding proteins for trans-activating other viral genes or regulating the host's cell cycle, and E2 harbors genes for viral DNA replication, while E3 and E4 genes play roles in modulating host immune responses (Kojaoghlanian et al., 2003) or inhibiting host cell apoptosis (Jornot et al., 2001). The late genes encode proteins for either the viral capsid or gene regulation (Cao et al., 2004).

Cellular receptors are required for efficient transduction by Ad. The coxsackie-adenovirus receptor (CAR) is the primary receptor, and the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are the secondary receptors for Ad to gain entry into a host cell (Parks, 2000). Infection initiates via attachment of the fiber knob to the CAR and subsequent binding of the penton base proteins to the integrin receptors, which allow a virus to enter the cell via receptor-mediated endocytosis. Ads can also enter cells through CAR-independent transduction, via heparin sulfate glycosoaminoglycans (Smith et al., 2002; Waddington et al., 2008). Following endocytosis, the virus escapes from the endosome through the lysis of the endosomal membrane and enters the nuclear pore complex via microtubule-mediated translocation. For a wild type Ad, transcription and replication begin upon entering the nucleus. During a lytic life cycle, viral DNA is packaged into virions by self-assembly of the capsid proteins and the viruses are then released following the death of the host cell (Cao et al., 2004). For gene replacement therapy, however, replication-defective Ads are used and these viruses do not go through the lytic life cycle or cause the death of host cells. Ad DNA does not integrate into the genome of the host cell and therefore, poses virtually no risk of insertional mutagenesis to the host cell. Unlike plasmids, the Ad genome is highly stable in transduced cells, making it attractive to be used for gene delivery (Benihoud et al., 1999; Ehrhardt et al., 2003).

Ad vectors are the most efficient class of vectors in terms of delivering genes into both dividing and non-dividing cells. Adenoviruses have been widely used as tools for gene delivery also because of their large capacity for incorporation of foreign genes and can be easily grown to high titers (Parks, 2000; Cao et al., 2004). The early Ad vectors were developed by deleting the E1 region that is required for viral DNA replication, and the E1 function was provided *in trans* by cells used for viral propagation during viral vector production. Ad vectors with E1 deleted can carry only 4 kb foreign DNA. To increase the cloning capacity, other Ad vectors were developed by deleting more than one early region (Parks, 2000). These Ad vectors have been used in a variety of experiments on gene transfer *in vitro* and *in vivo*.

Recombinant adenovirus with a tropism for airway cells initially appeared to be promising for airway gene transfer, but limitations associated with its use were subsequently identified in *in vivo* studies. Phase I gene therapy trials with the first generation of Ad vectors in patients with cystic fibrosis (CF) concluded that the level of gene transfer and expression is too low to achieve clinical benefits. CF, the most common monogenic fatal disorder in the Caucasian population, is caused by recessive mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (Rommens et al., 1989). CFTR is a cAMP-regulated chloride channel, and defective or absent CFTR in epithelial cells of many internal organs, including the lung, pancreas, intestine, gall bladder and reproductive organs, results in salt and water imbalance across the epithelium (Boucher, 1994; Tsui, 1995; Zabner et al., 1995). Although the disease affects multiple

organs, lung failure due to chronic infection and inflammation is currently responsible for most morbidity and mortality (Koehler et al., 2001a). Therefore, CF gene therapy studies to date have been aimed at treating the pulmonary manifestations. When the cystic fibrosis gene was identified in 1989, therapy seemed around corner (Pearson, 2009). Two decades of research have revealed quite a number of obstacles to the development of CF gene therapy.

Although a number of mutant CFTR transgenic mouse models have been developed to understand the pathogenesis of the CFTR lung disease and also to develop novel therapeutic strategies to ameliorate the disease condition (Snouwaert et al., 1995), the mice with mutated versions of CFTR have few obvious lung problems and thus make them poor models of the disease (Guilbault et al., 2007). Recently, CF pig models with a defective CFTR gene were generated (Rogers et al., 2008). These animals do develop lung diseases similar to that observed in humans, including infection in the lung, inflammation, and accumulation of mucus in the airway. Moreover, the newborn CF pigs are less able to eradicate bacteria from their lungs (Stoltz et al., 2010). These new models will help us to understand CF in people and to develop gene therapy strategies for treating this lung disease.

An early trial (Zabner et al., 1993) showed adenoviral transfer of *CFTR* to human nasal epithelium, with correction of nasal transmembrane potential differences (PD). But this gene transfer was correlated with injury caused by the application device; similar results were reported later by Grubb et al. (1994). It was confirmed (Knowles et al., 1995) that there is no functional correction of nasal PD in patients resulting from adenoviral CFTR transfer in the absence of injury of the epithelium. Several other studies with Ad vectors showed CFTR gene transfer, but none demonstrated sustained CFTR expression (Crystal et al., 1994; Bellon et al., 1997; Harvey et al., 1999; Joseph et al., 2001; Perricone et al., 2001).

The human studies mentioned above as well as experiments with animals (Dai et al., 1995; Yang et al., 1995, 1996; Wilson et al., 1998) showed that recombinant Ad vectors delivered to the airways or to the circulation system intravenously induce potent host immune responses that limits both stable transgene expression and the possibility for vector readministration (Wivel et al., 1999). These strong anti-vector responses are largely attributed to viral particles in the inoculum (McCoy et al., 1995; Kafri et al., 1998) and the expression of viral proteins in transduced cells (Wivel et al., 1999). The transgene product itself, particularly when derived from a species different from the host, can also contribute to host immune responses (Tripathy et al., 1996; Michou et al., 1997; O'Neal et al., 2000).

To improve safety and efficacy of Ad vectors, the helper-dependent (or gutted) adenoviral (HD-Ad) vector was developed by deleting all the viral coding sequences, leaving only the viral inverted terminal repeats (ITRs) and packaging signal. The deletion of viral coding sequences indeed reduces host adaptive immune responses (Parks, 2000) and prolongs transgene expression (Morsy et al., 1998; Kim et al., 2001; Toietta et al., 2003) in addition to the expansion of the cloning capacity to ~36 kb. Recently, efficient transduction was also achieved in rabbit lungs using aerosol delivery formulated with lysophosphatidylcholine (LPC) to enhance the transduction efficiency (Koehler et al., 2005). Because HD-Ad vectors have the same capsid proteins, host immune responses to the vectors are still present (Morral et al., 2002; Brunetti-Pierri et al., 2004). Since, however, non-capsid proteins encoded by Ad (Schaack et al., 2004) can cause inflammation, the innate immune response to HD-Ad vectors is attenuated as demonstrated in gene transfer studies in mice (Morsy et al., 1998; Kim et al., 2001; Toietta et al., 2003). In addition, it has also been shown that readministration of HD-Ad vectors to mouse lungs can be achieved by using transient immunosuppression (Cao et al., 2011). For HD-Ad vector propagation, a helper virus is required to provide functions for DNA replication and assembly of the virions (Parks, 2000). One past problem in using the HD-Ad vector in clinical studies was the difficulty in large-scale production (Cao et al., 2004). This problem has recently been solved by Ng's group by using a new helper virus and 116 cells capable of growing in liquid suspension (Palmer and Ng, 2003).

Adeno-associated viral vectors

Recombinant adeno-associated virus (AAV) vectors are another major type of viral vectors currently used in gene therapy studies. AAV is considered nonpathogenic with low innate/inflammatory responses and this is the major advantage for using AAV in gene therapy (Hirsch et al., 2010). AAV is a replication-defective parvovirus that depends on a helper virus, either adenovirus or herpes virus, for its propagation during lytic infection (Berns and Giraud, 1996). AAV has a very small (about 4.7 kb) single-stranded DNA genome (Carter, 2004), including 145-bp ITRs that are the only sequences required for vector construction. The AAV genome encodes three viral capsid proteins (VP1, VP2 and VP3), Rep68/78 proteins that bind to the ITRs and mediate its integration into the host chromosome (Carter, 2004), and Rep52/40 proteins whose functions are not clear (Aiuti and Roncarolo, 2009). Therefore, AAV vectors are essentially helper-dependent viral vectors retaining only the ITRs. All the capsid and Rep proteins are provided *in trans* by either a helper virus or sequences integrated into the host genome during vector propagation. The small cloning capacity (4.5 kb) is a disadvantage of AAV vectors because it limits their utilization for therapeutic genes with coding sequences longer than 4.5 kb and with little room for inclusion of DNA regulatory elements even for smaller genes.

AAV uses heparin sulfate proteoglycan as receptor to gain

entry into host cells via endocytosis (Summerford and Samulski, 1998) and it can transduce both dividing and non-dividing cells. The viral genome can integrate into a host chromosome or stay episomally in the cell; the frequency of integration is very low, however (Hargrove et al., 1997). In non-dividing cells, AAV vectors stay episomally as head to tail concatemers (Schnepp et al., 2003). While the wild type AAV integrates specifically at the AAVS1 site on human chromosome 19 (Kotin et al., 1990), AAV vectors integrate randomly (Kearns et al., 1996) at a much lower frequency than previously believed (Carter, 2004). Although AAV can transduce a broad spectrum of cell types, a very high ratio of viral particles is required to transduce target cells. This may be partially due to the low levels of receptor present on the cell surface; for AAV integration or gene expression, the single-stranded DNA genome has to be converted into double-stranded and this could also be a rate-limiting step especially in non-dividing cells or primary cultured cells (Aiuti and Roncarolo, 2009). There are 9 serotypes of AAV identified (AAV1 to AAV9) and some of them show differences in tissue-tropism (Gao et al., 2002). For example, AAV6 (Blankinship et al., 2004) and AAV8 (Gao et al., 2002) transduce muscle cells very efficiently. In addition, levels of neutralizing antibodies against them are different; sera from humans show little neutralizing activity to AAV7 and AAV8. Since the viral capsid proteins can be exchanged, a desired serotype can be selected during vector production to maximize the efficiency of gene delivery to a particular tissue. Serotype-switching can also be used to minimize neutralizing antibodies during vector readministration.

Since the cloning capacity of AAV is limited, inclusion of extra promoter/enhancer elements for better transgene expression would further reduce the capacity. This problem can be minimized by reducing the size of a therapeutic gene. One example was the construction of an AAV vector for expression of a minidystrophin gene for gene therapy against Duchenne muscular dystrophy (DMD) (Wang et al., 2000). DMD is an X-linked, fatal genetic muscle disease affecting 1 of every 3500 males born and the progressive muscle degeneration leads to death of patients by their early twenties. The dystrophin gene spans nearly 3 million bp on the X-chromosome (Koenig et al., 1987) and it produces a mRNA of 14 kb. Wang et al. created a 4.2-kb minidystrophin gene and cloned it into an AAV vector containing a muscle-specific creatine kinase (MCK) promoter. They showed that this vector effectively ameliorates muscular dystrophy in the mdx mouse model (Wang et al., 2000). The other example was the development of an AAV vector expressing a mini version of the *CFTR* gene (Sirninger et al., 2004). Since viral promoters are often attenuated or silenced, the beta-actin promoter plus an enhancer from the cytomegalovirus (CMV) was used to drive a CFTR minigene. In addition to the utilization of minigenes, trans-splicing has been explored to expand the cloning capacity of AAV vectors. The process of trans-splicing was initially discovered in *Trypanosoma brucei* 23 years ago when different surface glycoprotein mRNAs were found to carry a common 39-nucleotide sequence, namely, the spliced leader sequence (Boothroyd and Cross, 1982). In trans-splicing, two primary RNA transcripts are used to produce a mRNA by the RNA splicing machinery and this subject has been reviewed (Liang et al., 2003). Two AAV vectors can be used to produce two half transcripts for a therapeutic gene and the transcripts can be trans-spliced in cells to produce a functional mRNA (Yan et al., 2000; Duan et al., 2001; Reich et al., 2003; Pergolizzi and Crystal, 2004).

CF gene therapy has yielded mixed results with AAV vectors. The major advantage of these vectors is that they are less immunogenic than Ad vectors (Wagner et al., 1999), but since the AAV vector used in this study contains only the promoter in the ITR sequence to drive *CFTR* expression, limited gene expression was detected when the maxillary sinus of CF patients was used as a delivery test site (Wagner et al., 1999). The first multi-dose inhalation trial in CF patients demonstrated safety and a transient improvement of lung function (Moss et al., 2004). Clinical studies in other organ systems also showed gene transfer and expression (Aiuti and Roncarolo, 2009), and major breakthroughs using AVV vectors in clinical studies on CF patients remain to be achieved. The recent discovery of AAV9 and AAV8 being able to cross blood-brain or blood vessel barriers efficiently transducing brain tissues or skeletal and heart muscles (Forsayeth and Bankiewicz, 2011) will facilitate the utilization of AAV vectors in clinical applications.

Pre-clinical and clinical studies with AAV vectors have shown the potential of gene therapy for treating eye diseases such as Leber congenital amaurosis (LCA) (Cideciyan, 2010; Herzog et al., 2010; Stein et al., 2011). LCA is a rare form of hereditary retinal degeneration caused by mutations in more than a dozen genes. *RPE65*, one of these mutated genes, is highly expressed in the retinal pigment epithelium and it encodes an isomerohydrolase that is required for converting all-*trans*-retinyl esters into 11-*cis*-retinal, the natural ligand and chromophore for the opsins of rod and cone photoreceptor cells. Without 11-*cis*-retinal, the opsins neither capture nor transduce light into electrical responses initiating vision. Loss of RPE65 function disrupts the visual cycle, causes severe and progressive photoreceptor degeneration and ultimately results in blindness. Preclinical studies with AAV2 vector demonstrated restoration of vision upon RPE65 gene transfer into RPE cells in mice and dogs (Acland et al., 2001; Li et al., 2011). In clinical trials, three groups reported success in vision improvement for patients with RPE65-associated LCA. Patients gained vision as evidenced by behavior correlates with the ability to walk through the maze (Bainbridge et al., 2008; Cideciyan et al., 2008; Maguire et al., 2008). More recent studies showed a correlation between the visual field maps and the distribution of cortical activations for the treated eyes (Ashtari et al., 2011). It is clear that

LCA-RPE65 gene therapy can help restore, to a degree, the visual function of both rods and cones.

Retroviral vectors

Retroviral vectors are based on retroviruses which comprise a large class of enveloped viruses with two identical single-stranded RNAs (7–11 kb) as the viral genome. The retroviridae family consists of seven genera: alpharetrovirus, betaretrovirus, gammaretrovirus, deltaretrovirus, epsilonretrovirus, lentivirus and spumavirus (Pringle, 1999). The first five genera were also known as oncoretroviruses, and the first retroviral vector was developed based on a gammaretrovirus, Moloney murine leukemia virus (MoMLV). Retroviral vectors were also developed based on lentivirus (Copreni et al., 2004) although traditionally only vectors based on oncoretroviruses were referred to as retroviral vectors. The retroviral genome contains three genes (*gag* coding for the group specific antigens or core protein, *pol* for reverse transcriptase and *env* for viral envelope protein), two long terminal repeats (LTRs) and a sequence required for packaging viral RNA during viral propagation. During the infection, the retroviral envelope protein interacts with a cell surface receptor to gain entry into the host cell. Different viruses use different receptors. For example, MoMLV uses a sodium-dependent phosphate transporter as its receptor while lentivirus uses CD4 as its primary receptor (Overbaugh et al., 2001). Upon entering a host cell, the RNA genome is reverse-transcribed into double-stranded DNA that binds to cellular proteins to form a nucleoprotein preintegration complex (PIC) that migrates to the nucleus and integrates into the host genome. The nuclear membrane, however, can be a barrier for some retroviruses. For instance, the PICs of MoMLV cannot cross the membrane and require a mitotic cycle to disrupt the nuclear membrane for the viral genome to reach the nucleus (Barquinero et al., 2004). Therefore, MoMLV cannot transduce non-dividing cells. On the other hand, lentiviral vectors based on human immunodeficiency virus-1 (HIV-1) do not have this limitation and thus can transduce non-dividing cells (Barquinero et al., 2004).

For development of retroviral vectors, only the 5′ and 3′ LTRs as well as the packaging signal sequence are required for the vector DNA, while the functions of *gag*, *pol* and *env* are provided by host cells used for viral propagation. Over the years, there were several types of improvements made for the retroviral vectors. The first type was the improvement in transgene expression. This was done by using LTR variants for transgene expression in certain cell types (Smith, 1995) and engineering regulatory regions to enhance transgene expression or reduce transcriptional silencing in specific target cells (Barquinero et al., 2004). Hybrid retroviral vectors with improved expression in hematopoietic cells were developed using sequences from the murine embryonic stem cell virus and the Friend mink cell focus-forming virus or the myeloproliferative sarcoma virus (Baum et al., 1995). Challita et al. (1995) increased transgene expression and decreased DNA methylation of retroviral vectors by introducing multiple changes in the LTRs.

The second type of improvement was the utilization of alternative envelope proteins, a technique called *pseudotyping*. For example, many hematopoietic cells express higher levels of Glvr1, the receptor for the gibbon ape leukemia virus (GALV), than that of Ram 1 the receptor for amphotropic vectors (Bauer et al., 1995). GALV-pseudotyped vector particles are more efficient at transducing primate repopulating hematopoietic stem cells than conventional amphotropic vector particles. Another example is the feline endogenous retrovirus (RD114)-pseudotyped vector particles that were shown to be more efficient in transduction of cord blood cells than amphotropic vector particles (van der Loo et al., 2002). In addition, although the vesicular stomatitis virus-G (VSV-G)-pseudotyped oncoretroviral vector particles did not show enhancement in transducing primitive primate hematopoietic cells (Evans et al., 1999), VSV-G-pseudotyped lentiviral vectors could be useful for gene delivery to other cell types such as airway epithelial cells (Sinn et al., 2003; Copreni et al., 2004). VSV-G is a fusogenic protein that interacts with membrane phospholipids to facilitate transduction, and VSV-G-pseudotyped vector particles are more stable (Yam et al., 1998).

The third type of improvement in retroviral design was the development of self-inactivating (SIN) vectors. A deletion of 229 bp in the 3′ LTR eliminated the enhancer and promoter present in the LTR (Yu et al., 1986). During the reverse transcription, the deletion was transferred to the 5′ LTR, resulting in a vector without viral promoters or enhancers. The absence of the viral promoter and enhancer minimizes the risk of activation of oncogenes as a result of integration and allows transgene expression under the control of desired promoter/enhancer sequences. Moreover, it is possible to reduce insertional mutagenesis in conjunction with zinc-finger nuclease technology in lentiviral vectors to allow for site-specific gene correction or addition to predefined chromosomal loci (Mátrai et al., 2010). The most important improvement needed right now is to design a vector that can be safely and specifically integrated at a chromosomal site that allows efficient therapeutic gene expression.

In addition to the improvements made in retroviral vector development, other strategies have also been used to enhance the retroviral gene delivery. Since retroviral vectors have been heavily used for *ex vivo* gene therapy to target hematopoietic stem cells, several techniques were developed to enhance their transduction efficiency. The first strategy was to use cytokines to mobilize the primitive stem cells to peripheral blood and to improve the susceptibility of the cells to retroviral transduction. It was shown that in mice and monkeys, treatment of donor animals with granulocyte colony-stimulating factor (G-CSF) and stem cell factor

(SCF) increased the number of CD34+ cells targeted for gene transfer in both peripheral blood and bone marrow, and these cells could be more efficiently transduced (Bodine et al., 1996; Dunbar et al., 1996). The second strategy was to use cytokines to induce the primitive human hematopoietic cells to divide, therefore enhancing the retroviral transduction efficiency while maintaining the hematopoietic potential. Primitive human hematopoietic cells could be stimulated to undergo self-renewal while retaining repopulating potential when SCF and Flt-3 ligand were used in combination with IL-3, IL-6, G-CSF (Glimm and Eaves, 1999). These stimulated cells could be transduced more efficiently (Veena et al., 1998). The third strategy was to use fibronectin fragments to enhance retroviral transduction efficiency (Hanenberg et al., 1996; Moritz et al., 1996). Fibronectin fragments bind both vector particles and target cells and therefore may facilitate the uptake of vector particles by the cells (Moritz et al., 1996).

In early 2000, three human gene therapy studies successfully brought clinical benefits to patients and all these trials were conducted by using retroviral vectors. The first trial was reported (Cavazzana-Calvo et al., 2000) in the treatment of children with X-linked severe combined immunodeficiency disease (SCID-X1). The patients have a defective gene encoding the common gamma chain (γc) of receptors for IL-2, -4, -7, -9, -15 and-21, which leads to the absence of functional B, T and NK cells, and they die at very early age. Ten patients were treated by reinfusion of their own CD34+ bone marrow cells transduced with a retroviral vector expressing the wild-type γc gene in the absence of any myelosuppression. Myelosuppression means a decrease in number of blood cells produced from the bone marrow following a pharmacological intervention or under a diseased situation. Nine of ten patients showed almost normal levels of T-cell counts and significantly improved immune function. Despite the insertional mutagenesis resulting in activation of the T-cell proto-oncogene LMO-2 in two patients (Hacein-Bey-Abina et al., 2003), this clinical trial was the first milestone to mark the feasibility of using gene therapy to cure a human disease. The second successful clinical trial was conducted in two children with adenosine deaminase-deficient severe combined immunodeficiency (ADA-SCID) (Aiuti et al., 2002). In ADA-SCID patients, the accumulation of purine metabolites toxic primarily to T cells leads to the immunodeficiency. Autologous CD34+ cells from patients were transduced with a retroviral vector (GIADA1) and, four days later, reinfused into the patients, who received two doses of busulfan (2 mg/kg per day) for transient myelosuppression prior to the reinfusion. During the follow-up for more than one year, two patients were in good clinical conditions and did not experience any severe infectious episodes. The third case was reported in the UK (Gaspar et al., 2004) on four SCID-X1 patients who received treatment similar to that reported in the first case (Cavazzana-Calvo et al., 2000).

These early clinical trials using retroviral vector gene transfer have led to immune restoration in SCID-X1and ADA-SCID patients. Although severe complications appeared in several SCID-X1 patients in whom the integrated retroviral vectors led to leukoproliferative disorders, retroviral transduction of hematopoietic stem cells showed high therapeutic efficiency in ADA-SCID patients without development of leukemia. More than 30 SCID-X1 patients have benefited from gene therapy, achieved life-saving immune reconstitution lasting for up to 10 years following the treatment (Aiuti and Roncarolo, 2009). Eight of ten ADA-SCID children have essentially been cured, living normal lives and no longer requiring ADA enzyme replacement (Aiuti et al., 2009). The newest viral vectors in clinical trials are HIV-based lentiviral vectors, in which all HIV genes have been deleted. Currently, several clinical trials are designed with self-inactivating lentiviral vectors to treat SCID. The development of novel strategies based on zinc-finger nucleases that can correct specific DNA sequences is very promising but not yet ready for clinical applications (Aiuti and Roncarolo, 2009).

Nonviral vectors

Nonviral methods for gene delivery have also been extensively explored over the past two decades in searching for alternatives safer than viral gene delivery. Liposomes are the most studied types of nonviral vectors. Based on their charge, liposomes can be classified into two classes, positively charged or cationic liposomes and negatively charged or pH-sensitive liposomes (Singhal and Huang, 1994). Cationic liposomes are commonly used for gene delivery and many types of formulations are available. They are made up of a cationic lipid and a neutral lipid, often dioleoylphosphatidylethanolamine (DOPE) or cholestrol. There are many types of cationic lipids that have been tested in gene delivery studies, such as 3β[N(N′,N′-dimethylaminoethane) carbamoyl)-cholesterol (DC-chol), 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP), and N-[2,3-(dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride (DOTMA). Detailed information regarding various cationic liposomes was covered by Gao and Huang (Caplen et al., 1995). Negatively charged or pH-sensitive liposomes contain DOPE and another lipid, such as palmitoylhomocysteine or free fatty acids. They fuse with other lipid bilayers at low pH and can be used to deliver molecules to cytoplasm. This type of liposomes was less frequently used for gene delivery (Singhal and Huang 1994). Although liposomes have been commonly used for delivery of DNA to cultured cells, their potential for *in vivo* gene delivery remains to be shown. Other types of nonviral formulations, such as those using polyethyleneimine (PEI) or polylysine, were also studied for their gene transfer ability (Kollen et al., 1999; Bragonzi et al., 2000). They share with liposomes the same problem of inefficient gene transfer *in vivo*.

Many clinical trials have been conducted with cationic liposomes mostly to assess the potential for treating patients

with CF. The first liposome CF trial was carried out in the noses of CF patients (DF508 mutation) using DC-Chol:DOPE complexed to *CFTR* cDNA (Caplen et al., 1995). Later, several nasal trials with various formulations of liposomes were performed (Bellon et al., 1997; Porteous et al., 1997; Zabner et al., 1997). The absence of viral proteins predicts that non-viral vectors should be less immunogenic and therefore capable of readministration. However, only one clinical trial appears to have evaluated the outcome of multiple dosing of nonviral vectors in nasal epithelia of patients with CF (Hyde et al., 2000; Gill et al., 2010). Although safe gene transfer and/or a statistically significant partial correction of nasal electrophysiology was reported in these trials, further improvement is needed to achieve the level of efficacy needed for CF gene therapy. A double-blind placebo-controlled nasal trial with p-ethyl-dimyristoylphosphadityl choline (EDMPC) complexed with human *CFTR* cDNA concluded that this lipid-DNA complex was also relatively safe but did not produce evidence of gene transfer to the nasal epithelium by physiological or molecular measures (Noone et al., 2000). The first clinical trial in lung was carried out with *CFTR* cDNA complexed with GL67 liposomes (Alton et al., 1999). One week after nebulizing the DNA/liposome complexes into the lungs of eight subjects, seven of the eight developed mild flu-like symptoms that disappeared within 36 h. It was found later that DNA produced in bacteria contributes to the inflammatory process. Six of eight patients showed a small change in chloride conductance towards normal values. A trial later demonstrated that even eukaryotic DNA in combination with GL67 elicits immunogenic responses in CF patients (Ruiz et al., 2001). The newly developed CpG-free plasmid vector should reduce inflammation (Hyde et al., 2008). However for nonviral vector liposomes to be used in future gene therapy, it is essential to demonstrate their ability to achieve efficient gene delivery and sustained transgene expression in animal models that remain to be shown.

OBSTACLES AND SOLUTIONS

Despite all the advancements in the development of vectors for gene delivery and a few successful gene therapy trials, fundamental problems in gene delivery and transgene expression remain to be solved before gene therapy can be used as a common practice in hospitals. Over the years, many of these problems have been identified. In the following, we shall use gene delivery to the lung airway as an example to point out the obstacles, including physical barriers to gene delivery, host immune responses and maintaining transgene expression, and to propose solutions to overcome these obstacles.

Physical barriers to gene delivery

Delivery of genes to airway epithelium for therapeutic purposes seemed easy at first, because lung epithelial cells interface with the environment and are therefore accessible. However, problems encountered in airway gene delivery were more substantial than originally expected. The lung airway represents an attractive target for gene transfer because vectors can be delivered efficiently to the airway surface. The lung epithelium is a continuous layer of cells lining all the airways and air spaces from the trachea to alveoli, and it is composed of at least eight different cell types that have a range of functions (Spina, 1998). Lung airways provide important defense capabilities to keep infectious or harmful particles from entering the body through the respiratory system (Koehler et al., 2001a), in addition to providing a passage for air to go in and out. The surface layer of mucous-containing liquid produced from epithelial goblet cells and submucosal glands can bind foreign particles that are then cleared from airways by the sweeping action of ciliated epithelial cells (Boucher, 1999). The intercellular space of the airway epithelial cells is linked by tight junctions so that the entire epithelium forms a physical barrier keeping infectious particles from penetrating the surface layer of the airway (Boucher, 1999; Koehler et al., 2001a). The airway epithelium has also been suggested to play an important role in mucosal immunoglobulin A production, through supplying cytokines responsible for B-cell isotype switch, growth and differentiation into IgA-secreting plasma cells (Salvi and Holgate, 1999). Additionally, lung epithelial cells are important in maintaining ion and water homeostasis (Boucher et al., 1986; Boucher, 1994), as evident from diseases such as CF (Rommens et al., 1989; Tsui, 1995). The alveolar epithelial cells are mainly responsible for air exchange or producing surfactants to keep the airspace open. Macrophages are the major type of non-epithelial cells in the airway and they comprise of more than 90% of the cells present in bronchoalveolar lavage (BAL) fluids (washout fluids from airways and alveoli) (Zsengellér et al., 2000).

When genes in viral or nonviral vectors are delivered to the lung airway, they can be trapped by the layer of mucous and swept out by the mucociliary action. The transmembrane mucin MUC1 as well as other sialoglycoconjugates was reported to inhibit Ad-mediated gene transfer to epithelial cells (Arcasoy et al., 1997). Consistent with these results, glycocalyx on the apical surface of polarized epithelial cells was found to act as a barrier to Ad-mediated gene transfer (Pickles et al., 2000). The mucous layer on the surface of cultured epithelial cells was also found as a barrier to AAV-mediated gene transfer (Bals et al., 1999). One strategy to overcome the problem of the mucous layer barrier, as suggested by Koehler et al. (2001a) is to use mucolytic reagents prior to vector delivery. The second strategy is to neutralize the vector trapping sites on presumably negatively charged mucins, or glycosaminoglycans. Interestingly, Kaplan et al. showed that various polycations, such as DEAE-Dextran, polylysines, polybrene, protamine and

branched polyethylenimine, could enhance Ad transduction in mouse airways (Kaplan et al., 1998). These polycations most likely neutralize the viral vector trapping sites in the airway. The polycations with no side effects in human, such as DEAD-Dextran (Pupita and Barone, 1983), may be used to reduce the viral vector particles used in gene transfer, thus decreasing host immune responses in lung gene therapy.

The second physical barrier is the loss of gene therapy vectors (viral or nonviral) to lung macrophages. For example, it was demonstrated that 70% of adenoviral vectors were lost in mouse airway within 24 h and that depletion of macrophages with liposome/dichloromethylene-biphosphonate (Cl_2MDP or clodronate) complexes resulted in a 100% increase in vector DNA recovered from the lung (Worgall et al., 1997). The mechanism of vector uptaking by macrophages is likely different from that involved in cell transduction because macrophages are hardly transduced despite they take up Ad vectors very efficiently (Zsengellér et al., 2000). Since lung macrophages not only destroy gene therapy vectors, but also play a major role in the innate immune response (this will be addressed later), it is important to consider them as a problem in lung gene therapy. One strategy to minimize the problem is to transiently deplete lung macrophages. Gadolinium chloride (Singh and de la Concha-Bermejillo, 1998) or liposome-encapsulated Cl_2MDP (Thepen et al., 1991; Worgall et al., 1997) were used to deplete lung macrophages in animals. But, there is no such a drug identified yet for depletion of human airway macrophages. The second strategy is to avoid macrophages. For example, when vectors delivered by aerosol, large size aerosol droplets or particles will preferentially deposit in the airway instead of alveoli where macrophages are abundant. Finally, pharmacological intervention may be used to block the macrophage function. It was recently reported that lung macrophages of knockout mice lacking GM-CSF expression or transcription factor PU.1 activity are incapable of taking up Ad vectors (Berclaz et al., 2002). Therefore, reagents may be developed to block the GM-CSF or PU.1 prior to gene delivery.

The third physical barrier is the lack of viral receptors present on the apical surface of airway epithelial cells for efficient transduction. For example, the major receptor for Ad, CAR, is expressed on the basal lateral side where the virus cannot reach unless the tight junction is loosened. One strategy is to modify viral vectors so that they can recognize receptors expressed on the apical surface. This can be done by “pseudotyping” the vectors using different capsid proteins (Ad or AAV) or envelop proteins (Retrovirus) or artificially modifying these proteins so that they can recognize the receptors on the apical side (Barnett et al., 2002). The other strategy is to use reagents to transiently break the tight junctions. For example, application of the Ca^{2+} chelator, EGTA to airways prior to vector delivery, can enhance Ad-mediated gene transfer significantly (Chu et al., 2001). In addition, L-α-lysophosphatidylcholine (LPC) can also achieve the same effect in mice (Limberis et al., 2002) and, as shown by my group, LPC can be mixed with Ad vectors for aerosol delivery to rabbit airways (Cao et al., 2005). Finally gene therapy vectors can be lost in other ways such as degradation or inactivation by enzymes secreted to the airway surface fluid. Even delivered inside a cell, plasmid DNA can be quickly degraded by intracellular nucleases. These problems were extensively reviewed by Koehler et al. (2001a).

Immunological barriers

The interaction between viral vector and airway epithelial cells initiates multiple innate and adaptive immune responses resulting in the production of antiviral substances, including type I and type II interferons, lactoferrin, β-defensins, and the production of cytokines and chemokines, which recruit inflammatory cells and influence adaptive immunity. Although the adaptive immune response to viral vectors was recognized early on and investigated extensively (Dai et al., 1995; Kay et al., 1995; Yang et al., 1995), the innate immune response was greatly underestimated. This was evident from the first incidence of gene therapy related death. In September 1999, a patient who received a high dose of an Ad vector via the hepatic artery succumbed to the acute toxicity caused by the vector. The news shocked the scientific community. Post-mortem analysis confirmed that the patient suffered from systemic inflammation, biochemically detectable disseminated intravascular coagulation, and multiple organ failure within 98 h (Raper et al., 2003). Clearly this was the result of the patient’s innate immune system reacting to the high dose of the Ad vector.

Innate immune response

The innate immune system is phylogenetically conserved and is present in almost all multicellular organisms (Hennemann et al., 1999). It is the first line of defense against invading pathogens through recognition of conserved microbial structure or products known as pathogen-associated molecular pattern (PAMPs) by a set of receptors called pattern recognition receptors (PRRs) (Huang and Yang, 2009). These receptors are inherited, therefore limited in numbers, and expressed on many cell types involved in the innate immune system, especially, on macrophages, dendritic cells, and B cells. They can be functionally classified into three categories: secreted, endocytic and signaling. The secreted receptors, such as the mannan-binding lectin, function as opsonins by interacting with their ligands flagging the microorganisms for recognition by the complement system and phagocytes. The endocytic receptors expressed on the surface of phagocytes, such as the macrophage scavenger receptor, mediate the uptake and delivery of pathogens into lysosomes where they are destroyed (Suzuki et al., 1997). The pathogen-derived peptides are then presented by

major-histocompatibility-complex (MHC) molecules on the surface of macrophages. The signaling receptors, such as Toll-like receptors, interact with their ligands and activate signal-transduction pathways for expression of a variety of cytokines.

For airway gene delivery, the innate immunity mediated by phagocytes (for example, lung macrophages) that quickly engulf vector particles is the first obstacle to gene transfer. The macrophages not only take up gene therapy vectors and destroy them, but also initiate the production of proinflammatory cytokines through interacting with lung epithelial cells (Lee et al., 2010). Phagocytic cells and epithelium express Toll-like receptors (TLRs) and RIG-I-like (retinoid-inducible gene-1-like) receptors, which recognize nucleic acid and proteins derived from viral pathogens including viral DNA, single-stranded RNA and double-stranded RNA (Sinn et al., 2009). Inflammatory cytokines, such as TNF-α, IL-6, MIP-2 and MIP-1α, were dramatically induced in macrophages upon Ad vector delivery to mouse airways within 6 h (Zsengellér et al., 2000). The proinflammatory cytokines released from macrophages can affect other cells in the lung as well as macrophages themselves leading to a cytokine cascade, which could lead to airway damage, if the host cannot shut down the cascade. One of the important pathways involved in the induction of inflammatory cytokines is the NF-κB signaling pathway (Baldwin, 1996). A variety of inflammatory cytokines, such as IL-6 and IL-8, can be induced by the activation of NF-κB, which is inactive when it is associated with its inhibitor IκB in cytoplasm. When cells are induced with microbial products, such as lipopolysaccharide (LPS) or certain cytokines, such as TNF-α and IL-1ß, the signals are channeled inside the cell through a pathway and cause the activation of the IκB kinase (IKK) through phosphorylation. The activated IKK, in turn, phosphoylates IκB leading to its ubiquitination and degradation (Baldwin, 1996); therefore NF-kB is released from inhibition and translocated into nucleus resulting in up-regulation of its target genes. Even using nonviral vectors, bacterial DNA can be recognized by TLR 9 which activates the NF-κB pathway (Cao et al., 2004). Other phagocytic cell types, such as neutrophils and natural killer cells, are recruited in response to viral vector delivery. The infiltrated cells further produce inflammatory mediators. In humans, a high level of IL-8, a potent neutrophil chemoattractant, can cause neutrophil infiltration that leads to tissue damage (Matsushima et al., 1988; Cao et al., 2005). Roles of other cell types in the initial innate immune response to gene therapy vectors are not clear. Lung epithelial cells may produce IP-10 (Borgland et al., 2000), but very few other cytokines, in response to Ad vectors. The innate immune response not only leads to acute toxicity, but also controls the adaptive immune response.

Patients receiving Ad vectors often show an acute inflammatory response. Even for nonviral vector-mediated gene transfer, patients show inflammatory response (Ruiz et al., 2001). Therefore, it is very important to reduce the host innate immune response during gene therapy treatment. One of the strategies is to eliminate or inactivate lung macrophages as described earlier. Secondly, anti-inflammatory drugs, such as corticosteroids or Ibuprofen, can be used to reduce inflammation (Cao et al., 2004). If possible, vectors that do not cause strong host innate immune responses should be used. For example, AAV instead of Ad vectors should be used if they show the same efficiency in a particular gene delivery task.

Adaptive immune response

The adaptive immunity relies on T and B lymphocytes to produce cellular and humoral responses to infectious agents. During postnatal development, an extremely diverse repertoire of receptors is generated randomly and each type of receptors, that recognizes a unique antigen, is expressed on the surface of one lymphocyte only. The cells bearing useful receptors are subsequently selected from billions of lymphocytes for clonal expansion by interacting with antigens. Antigens are bound to the MHC II molecules on the surface of professional-antigen-presenting cells, normally macrophages or dendritic cells and presented to helper T cells. Activation of helper T cells by antigen presenting cells also requires a co-stimulatory signal, e.g., CD80 or CD86, on the surface of the antigen-presenting cell to bind to CD28 on the surface of the T cell. The expression of co-stimulatory molecules is regulated by innate immunity (Fearon and Locksley, 1996), and therefore, the adaptive immunity is controlled by the innate immunity. After activation, helper T cells control other cells in the adaptive immune system, such as activation of cytotoxic T cells to destroy infected cells and B cells to produce antibodies. Following elimination of an infection, some antigen-specific clones of T and B cells remain as "memory" lymphocytes so that the adaptive immune system remembers the antigens and destroys them quickly when encountered a second time. Compared to the innate immune response, the adaptive immune response is a slow process and it takes three to five days to mobilize enough lymphocytes to take action.

Previous work by several groups (Dai et al., 1995; Kay et al., 1995; Yang et al., 1995) showed clearly that both cellular and humoral responses are involved in Ad vector-mediated gene transfer in mice. Repeated delivery of viral vectors, or primary delivery to individuals with pre-existing immunity, is problematic because of antibodies against the capsid proteins. Various strategies can be used to overcome the problem. First of all, blocking the innate immune response may be used to reduce the adaptive immune response. Reducing the uptake of vectors by macrophages should inhibit antigen presentation. Therefore, all the strategies used to reduce the innate immune response mentioned above can be used to reduce the adaptive immune response. Another

strategy for effective repeated delivery of recombinant viruses is "serotype switching." (Mastrangeli et al., 1996) Gene therapy is initiated with one virus serotype, then switched to a second serotype for a subsequent administration, thereby avoiding neutralizing antibodies induced by the first serotype (Mack et al., 1997). However, the level and duration of transgene expression following serotype switching may be limited by cross-reactive cytotoxic T lymphocytes that can also target cells infected by the second serotype virus (Mack et al., 1997; Kaplan et al., 1998). Furthermore since all the viral gene therapy vectors used in the future will not express any viral coding proteins, transient immune modulation may be used to block the adaptive immune response. Drugs normally used for immunosuppression, such as cyclosporine, and cyclophosphamide, may be used to transiently modulate host adaptive immune responses. For example, it was shown that cyclophosphamide alone or in combination with cyclosporine A extended transgene expression mediated by the first generation adenoviral vector (Dai et al., 1995). Cyclophosphamide has also been shown to allow readministration of HD-Ad vectors with efficient expression of transgenes (Cao et al., 2011). Finally, blocking co-stimulatory pathways can be used to modulate the host adaptive immune response. Several groups showed that an antibody against CD40 ligand (Scaria et al., 1997; Wilson et al., 1998) or expressing CTLA4Ig, a fusion protein of cytotoxic T lymphocyte-associated protein 4 (CTLA4) and the Fc portion of immunoglobulin G (IgG), by the HD-Ad vector, improved transgene expression in rodents (Jiang et al., 2002; Yamashita et al., 2003).

Transgene expression barriers

How to control therapeutic gene expression was ignored initially by the gene therapy community since some viral promoters showed high activity in cultured cells. In addition, the initial goal in gene therapy research was to demonstrate gene transfer and gene expression. It was later discovered that viral promoters can be attenuated by host cytokines (Qin et al., 1997; Sung et al., 2001) and some retroviral promoters can be silenced following vector integration (Fearon and Locksley, 1996; Barquinero et al., 2000; Kalberer et al., 2000). It is well known now that the processes of gene transcription, RNA processing and mRNA transport are coupled. It is important to take into consideration all the DNA elements required for gene expression in the design of a gene expression cassette, such as enhancers for transcription, introns and polyA signals for RNA processing and transport as well as sequences for efficient translation and RNA stability. Some gene introns contain DNA control elements that are required to achieve cell-specific expression (Oshima et al., 1990; Aronow et al., 1992). In addition, for integration vectors, insulators or locus control regions are required to prevent transgene silencing due to the integration.

Cell-specific expression

The temporal and spatial expression of genes is determined by the DNA regulatory elements and gene positions on chromosomes. Ideally, for expression of a therapeutic gene, its own DNA regulatory elements should be used to drive the expression, since non-specific expression of a gene could result in adverse effects. Unfortunately, for many genes, their regulatory elements are not characterized. Initially, most gene therapy studies were done with promoters from viruses, such as Simian virus 40 (SV40), CMV and respiratory syncytial virus (RSV). As mentioned above, the viral promoters can be shut down in mammals by host cytokines although these promoters are quite active in cultured cells. Another problem associated with the viral promoters is that they are not cell or tissue-specific.

One strategy to solve this problem is to use DNA regulatory elements from a gene that show similar cell-specificity to that of a therapeutic gene. This strategy has been applied to the CF gene lung gene transfer by developing a gene expression cassette using DNA control elements from the human cytokeratin 18 gene which displays a very similar expression pattern as the *CFTR* gene (Chow et al., 1997, 2000). This gene expression cassette has been shown to efficiently express transgenes in HD-Ad vectors in mice (Toietta et al., 2003). It has been demonstrated that CF knockout mice treated with an HD-Ad vector expressing the *CFTR* gene from the K18 expression cassette became resistant, like the wild-type mice, to acute lung infection by a clinically relevant *Burkholdera Cepcacia* strain (Koehler et al., 2003). Recently, it has been shown that the K18 promoter-based HD-Ad vectors efficiently transduced airway epithelia of rabbits (Koehler et al., 2005). The other strategy is to use hybrid promoters with regulatory elements from different promoters. As mentioned early, an AAV vector using the beta-actin promoter plus an enhancer from the CMV promoter exhibited stronger expression of a *CFTR* minigene than that from vectors using viral promoters (Sirninger et al., 2004).

Sustained and regulated expression

Sustained therapeutic gene expression is critical for gene therapy. One major problem in CF lung gene therapy trials is the lack of sustained transgene expression. For liposome-mediated gene transfer, this can be attributed, at least partially, to the instability of the plasmid DNA in the transfected cells. For Ad vectors, host immune responses are the major problem. For AAV vectors, the small capacity for carrying DNA does not allow it to include enough DNA regulatory sequences. Since all the lung gene therapy trials conducted so far have not demonstrated efficient gene transfer, the lack of sustained transgene expression would further diminish any hope for showing clinical benefits. Therefore, solutions to the lack of sustained transgene

expression may be different for different vectors. For Ad vectors, simply switching to HD-Ad vectors can greatly improve the length of transgene expression in the lung (Toietta et al., 2003) and in other organs as well (Morsy et al., 1998; Kim et al., 2001; Maione et al., 2001). In addition, reducing the innate and adaptive immune responses will also enhance the sustained transgene expression if the therapeutic gene product was not previously present in patients. However, even for HD-Ad vectors to be used in lung gene therapy, there is an additional obstacle, epithelial cell turnover. This is also problematic for AAV vectors because their frequency of integration is very low (Schnepp et al., 2003). This requires readministration of vectors, which may not be as difficult as many think since the mucosal antibodies response is short-lived (Ahmed and Gray, 1996). For lentiviral vectors, unless the integration at a specific, benign chromosomal site can be established, they are too risky to be used for lung gene therapy, because of so many cells that need to be targeted. If integration vectors are used in gene therapy, all silencing elements should be removed and insulators or locus control regions may be added to prevent silencing caused by integration. We do not think that the current nonviral vectors can be used for efficient gene delivery to the lung and it is no use at this point to propose strategies to enhance sustained transgene expression for them.

Therapeutic genes must not only be expressed in the same tissues as the endogenous mutant genes but also follow their temporal expression patterns under different physiological conditions. The level as well as the temporal and spatial expression of each gene in eukaryotic organisms is carefully regulated. For example, some of the cytokine genes are expressed at a basal level, but their expression is induced under inflammation. Therefore, inflammation inducible expression is required for these genes if they are used for therapy. Recently, such an inflammation-inducible IL10 expressing HD-Ad was developed with a modified human IL-6 promoter. The production of IL-10 from this vector is shown to be highly inducible with TNF-α and IL-1β in cultured cells and with LPS in mice. The expression can be turned off upon withdraw of stimuli (Yang et al., 2010). For current gene therapy studies, most therapeutic genes are expressed from heterologous promoters. Modification of these promoters may be needed to make the therapeutic gene expression match the normal expression pattern and/or level. This type of fine-tuning of design will require comprehensive knowledge of gene regulation. This will still be a daunting task for years to come.

CONCLUDING REMARKS

Gene therapy was evolved largely from modern molecular genetics in anticipation of inventing novel treatments for human diseases. Our knowledge of gene expression and regulation plays a critical role in the design of gene therapy studies. Despite the initial underestimation of the difficulty in safety and efficiency of gene delivery, a few successful clinical studies have shown the feasibility of using gene therapy to cure fatal human diseases. Previous work in the field not only enhanced our understanding of the problems involved in gene therapy, but also will allow us to continue improving our methodology. Recent successful trials on the treatment of eye disease and inherited immune deficiencies are particularly encouraging and have raised hopes that human gene therapy as a standard treatment option will finally become a reality. Although the obstacles discussed here still impede our current progress in bringing clinical benefits of gene therapy to patients, all these problems can be solved theoretically. The complexity of human biology is expected to dictate different approaches to be used for different diseases, such as an *ex vivo* approach for blood diseases and a direct gene transfer method for airway diseases. Because of the differences in organ anatomy and in drug tolerance between rodents and humans, more large animal studies will be needed to check the safety and efficiency of gene therapy vectors before being clinically tested. As we know more about genes involved in diseases, more therapeutic "drugs" will be available. One can predict that more clinical benefits of gene therapy will be brought to the society by a combined effort of the persistent basic and clinical researchers in the field.

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ABBREVIATIONS

AAV, adeno-associated virus; ADA-SCID, adenosine deaminase-deficient severe combined immunodeficiency; CAR, coxsackie-adenovirus receptor; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CMV, cytomegalovirus; DMD, Duchenne muscular dystrophy; GALV, gibbon ape leukemia virus; GM-CSF, granulocyte macrophage-colony stimulating factor; HIV-1, human immunodeficiency virus-1; ITRs, inverted terminal repeats; LCA, Leber congenital amaurosis; RSV, respiratory syncytial virus; SCID-X1, X-linked severe combined immunodeficiency disease; SV40, Simian virus 40; TLRs, Toll-like receptors; VSV-G, vesicular stomatitis virus-G

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