Review

Potential of Helper-Dependent Adenoviral Vectors in Modulating Airway Innate Immunity

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Innate immune responses form the first line of defense against foreign insults and recently significant advances have been made in our understanding of the initiation of innate immune response along with its ability to modulate inflammation. In airway diseases such as asthma, COPD and cystic fibrosis, over reacting of the airway innate immune responses leads to cytokine imbalance and airway remodeling or damage. Helper-dependent adenoviral vectors have the potential to deliver genes to modulate airway innate immune responses and have many advantages over its predecessors. However, there still are a few limitations that need to be addressed prior to their use in clinical applications. *Cellular & Molecular Immunology*. 2007;4(2):81-89.

Key Words: adenovirus, gene therapy, innate immune response, cystic fibrosis

Innate immune responses and airway diseases

Airways not only function as a passage for air flow, but also as an innate immune organ against airborne bacteria, viruses or other harmful particles that are inhaled and deposited in them. Airways are equipped with all three types of fundamental innate immune defenses: anatomical and physiological barriers, cellular internalization, and inflammation. As the first type of airway defense, foreign substances can be trapped in the layer of mucous on top of the airway epithelium and swept out by mucociliary action. The layer of epithelial cells connected by tight junctions can also act as a physical barrier to foreign invaders. In addition to the role of physical barrier, airway epithelial cells synthesize products such as complement, collectins, lysozyme, lactoferrin, secretory leukocyte protease inhibitor, and defensins that can lead to localized destruction of invading microorganisms. The second type of airway defense involves leukocytes such as airway macrophages and neutrophils that can take up and destroy invading microorganisms and foreign substances. In addition to airway defense mechanisms, an inflammatory process can be orchestrated by airway cells including epithelial cells and leukocytes. The inflammation will result in production of more proinflammatory cytokines or chemokines, such as interleukin (IL)-8 and cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) (1), leading to recruitment of more neutrophils and other leukocytes to defend the airways.

Airway innate immunity requires the cellular integrity of airway epithelium and a balanced production of pro- and anti-inflammatory cytokines or chemokines. Deficiency in epithelial cells and/or inbalance of cytokine production can lead to airway diseases. A typical example is the cystic fibrosis lung disease (2). Cystic Fibrosis (CF) is the most common fatal genetic disease in Caucasian populations (~1 in 2,500 live births). CF is caused by recessive mutations of the *Cftr* gene, which encodes a transmembrane chloride channel expressed in the epithelium of multiple organs. Although multiple organs are affected in CF patients, major morbidity and mortality is a result of the CF lung disease. Classic CF lung disease presents with a progression of inflammation and infection, and a decline in lung function marked by mucopurulent plugging, bronchiectasis and intermittent bronchopneumonia. Although the mechanism by which *Cftr* mutations lead to CF airway disease is not completely understood, it has been shown that the airway fluid volume in CF lungs is reduced and this reduction is believed to impair the lung mucociliary function which is the first line of lung defense. In addition, CF epithelia have exaggerated responses to proinflammatory stimuli (3). It is likely that this combination of inefficient airway clearance and exaggerated inflammatory responses contributes to the excessive neutrophil infiltration in the CF lung. In other airway diseases, such as asthma or COPD, imbalance of cytokine/chemokine production is the major cause of the

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Table 1. Features of commonly used viral vectors in lung gene therapy studies

Viral vector	Integration into host genome	Length of expression	Difficulty in production	Immunogenicity	Insert size
HD Ad Vector	No	Long term	Low	Moderate	Up to 36 kb
FG Ad Vector	No	Up to 4 weeks	Low	High	10 kb
AAV	Yes – low frequency	Long-term	High	Low	5 kb
Lentivirus	Yes	Unknown	High	Unknown	8 kb

airway remodeling, which leads to clinical complications (4).

Since defects in airway innate immunity are caused by gene mutations or altered gene regulation, restoration of the airway innate immune response could be achieved, in theory, by using gene therapy. For example, gene replacement therapy has been attempted for patients with cystic fibrosis (5-8). In the case of exaggerated airway inflammation, genes encoding factors that downregulate inflammation, such as IL-10, can be delivered to the airway epithelial cells. Over the past few years, RNA interference (RNAi) has been shown to have great potential for clinical applications in knocking down target gene expression. RNA interference involves introduction of short interfering double stranded RNA with sequence specificity to a target gene (9-11). The small double stranded RNA can be chemically synthesized or expressed as a double stranded RNA with a hairpin like structure from a DNA vector delivered to target cells. Upon introduction of interfering RNAs in the cell, they are processed in the cytoplasm by an enzyme called Dicer (also a RNAIII endonuclease). Dicer recognizes dsRNAs, which could even be a hairpin structure, and cleaves them into short siRNAs, which are also double stranded and approximately 22 nucleotides in length. The siRNAs are then incorporated into a ribonucleoprotein complex known as RNA-inducing silencing complex (RISC) in a single stranded form. RISC identifies target RNA based on nearly perfect complementarity to the siRNA and cleaves it at a site near middle of complementarity. Therefore, the mRNA templates for the RNA being targeted are degraded.

In this review, we provide an overview of the challenges associated with the use of different vectors for airway gene delivery and in particular we highlight the advantages of using helper-dependent adenoviral vectors over its earlier predecessors in targeting gene delivery to the airways. The use of helper dependent system in delivering short interfering RNAs to downregulate inflammation is also highlighted along with the physical and immunological benefits and challenges of using this system.

For the last 10-15 years, there has been active research conducted on airway gene delivery and the vectors employed can be broadly divided into viral and non-viral vectors for gene delivery. Among the viral vectors, lentiviruses, adeno-associated viruses and adenoviruses have been actively investigated. Features of these viral vectors are summarized in Table 1. On the other hand, among non-viral vectors, cationic liposomes, glycoconjugates and transposon based integrating plasmid have been used as vectors for airway gene delivery.

Non-viral vectors for airway gene therapy

Cationic liposomes

Many cationic lipid/DNA complexes have been tested for *in vitro* and *in vivo* gene delivery ever since Lipofectin, a 1:1 mixture of cationic lipid DOTMA and colipid DOPE (12) was discovered in 1987. Cationic liposomes have been employed for gene delivery to the mouse lung, where gene expression up to 21 days was observed (13). Additionally, CFTR encoding plasmids have been delivered to the mouse lung using liposomes; some correction of the CF defect has been observed (14). However, a major drawback has been observed in gene delivery to larger animals, where gene expression cannot be observed for more than 7 days (15). Further analysis of safety profile in mice has also demonstrated a dose dependent increase in pulmonary inflammation (16). Clinical studies in humans have also shown that upon gene delivery to the nasal epithelium there is development of flu like symptoms and unfortunately, no vector mRNA could be detected (17). This highlights the two major limitations of gene delivery with cationic liposomes, first the toxicity and second its extremely low efficiency of gene transfer.

Glycoconjugates

Many receptors present on airway epithelium contain covalently linked carbohydrates, which can interact with lectins and result in vector uptake *via* receptor mediated endocytosis. The efficiency of glycoconjugate mediated gene therapy is determined both by specificity of the lectins expressed on cell surface along with lectins present inside cell which mediate intracellular trafficking (18). Glyco-conjugates have been tested for gene transfer both *in vitro* as well as *in vivo* models. Vectors encoding CFTR in complex with glycosylated polylysines have been administered to tracheal gland serous cells derived from CF patients with detectable CF expression (19). However, so far none of the studies have demonstrated both the efficiency of glyco-conjugates in mediating gene transfer, while simultaneously assesing the inflammatory and the safety profile of glyco-conjugates.

Transposon based integrating plasmids

Transposons are mobile DNA elements that can move across

genome and integrate at a site through the activity of integrase enzymes. The theoretical benefit of transposon mediated gene delivery is that the therapeutic gene can be expressed throughout the lifetime. In particular, sleeping beauty transposon system belonging to Tc1/mariner superfamily is being investigated for airway gene therapy, where the integration is mediated by a sleeping beauty transposase which can be provided to the cells as a plasmid or even as mRNA (20). One of the biggest limitations is the size of the genetic element to be delivered, delivery of genetic segments 6 kb or greater, reduces transfer efficiency by nearly 50% (21). Another drawback is the requirement for sustained transposase expression. Lung gene delivery studies in mice have shown that transgenic animals expressing transposase show sustained transgene expression, whereas in wild type animals expression could not be seen beyond 3 months (22). Similar to glycoconjugates, a safety profile for transposons has not been established and preliminary studies have shown that sleeping beauty transposons can mediate cancer development in wild type mice due to high rate of mobilization (23), reflecting a severe limitation with this approach for use in airway gene therapy.

Viral vectors for airway gene therapy

Lentivirus

Lentivirus belongs to the family *Retroviridae* and possesses a single stranded RNA genome, which upon entry into the cell is reverse-transcribed by the viral packaged reverse transcriptase. Prior to the development of lentiviral systems, retroviruses were extensively investigated; however, studies were quick to report that the efficiency of retroviruses was dependent on the differentiation and mitotic state of the cell, with highest transduction efficiency achieved with maximum differentiation and almost no transduction in fully differentiated epithelium (24). Therefore, due to limitations associated with retroviral vectors, lentiviral vectors in particular the ones derived from HIV (human immuno-deficiency virus) and FIV (feline immunodeficiency virus) were investigated for airway gene therapy. In contrast to retroviral vectors, VSV-G pseudotyped HIV based vectors could efficiently transduce non-dividing epithelial cells *in vitro* (25). At the same time, the vector could be used to efficiently transduce undifferentiated CF derived epithelial cells in human bronchial xenograft model (26), resulting in correction of the defect. However, one limitation was that these vectors could only transduce airway epithelia *in vivo* when access was provided to the basolateral surface by opening the tight junctions (27) through the use of agents such as lipophosphotidylcholine (LPC) (28). Due to this requirement, a lot of research is being conducted to pseudotype lentiviral vectors with proteins that can mediate efficient gene delivery to the airway epithelium. Pseudotyping of HIV vector with envelope protein derived from Zaire strain of Ebola has been shown to result in efficient transduction of airway epithelial cells *in vitro* as well as *in vivo* without a requirement for opening tight junctions (27). However, the biggest concern is activation of oncogene due to random integration of the viral vector (29), which clearly needs to be addressed prior to its widespread clinical applications.

Adeno-associated virus

Adeno-associated virus belongs to the dependovirus genus and contains a non-pathogenic single stranded DNA genome. It requires a coinfection with a helper virus such as adenovirus or herpes-simplex virus to complete its lytic life cycle (30). It was one of the earliest vectors used for CF gene therapy and has been used successfully for CFTR delivery to mice as well as rabbits; CF gene expression has been observed for as long as 6 months (31). One of the major drawbacks is the limitation in the size of the transgene that can be packaged within the vector, and a second limitation is that although most studies indicate that AAV exists as an episome, there is some evidence suggesting that the vector may integrate randomly in a non-specific fashion (32). CFTR delivery using AAV has been conducted in rhesus macaques with gene expression seen for up to 6 months without any toxicity or inflammation (33). AAV vectors have also been tested in clinical trials and although the vector was tolerated well with no measurable adverse reaction, the period of vector prevalence was much lower than that observed in primate studies (34). Phase II clinical trials for CF gene therapy in CF patients demonstrated that although there was a change in nasal potential difference upon delivery of the first vector dose, subsequent rounds of delivery did not result in improvement (35). Concomitantly with second dose of vector delivery, there was a gradual increase in antibody titer (36).

Adenovirus

Adenovirus belongs to the family of double stranded DNA viruses; it has a linear genome of approximately 34-38 kb in size and has been extensively employed in airway gene therapy (37). The first set of adenoviral vectors were referred to as first generation vectors (FGAd) and had a deletion of E1, or E1 and E3 regions, which are normally required for viral replication and suppression of host immune responses respectively (38). Inspite of extensive success with these vectors for CF gene therapy in cell culture studies, *in vivo* studies resulted in inefficient gene delivery (39). This was due in part to the localization of CAR (Coxsackie and adenovirus receptor) used for adenoviral entry in cells for it localizes to the basolateral surface of airway epithelium and the second reason was the transient and a dose-dependent cellular inflammatory response (40). Clinical trials initiated with first generation adenoviral vectors for CF gene therapy demonstrated acute toxicity and inflammation in CF patients upon gene delivery (41). The immune response further led to clearance of transduced cells, thereby limiting the extent of gene transduction. Hence, second generation vectors were developed (42), which although were slightly better than first generation, still suffered from the problems of immune responses due to some leaky expression of viral late genes (43). Finally, third generation adenoviral vectors, also

referred to as gutless or helper-dependent adenoviral (HDAd) vectors were developed. These vectors do not contain any adenoviral sequences except for 3' and 5' inverted terminal repeats and a packaging signal (44).

HDAd vectors for transgene expression or gene knockdown

HDAd vectors are the third generation of adenoviral vectors and are also referred to as gutless vectors because all viral encoding sequences are deleted, allowing for an increased cloning capacity of up to 36 kb. However, since adenovirus efficiently package DNA which is only 75-105% of Ad genome, and transgenes rarely reach 36 kb in size, stuffer DNA was used for designing vector DNA of appropriate size (45). Initially, lambda phage DNA was used as stuffer DNA. However, upon administration of gutless vectors, there was a cellular immune response, owing to the immunogenicity of the peptides encoded by stuffer DNA which is presented on cell surface (46). Therefore, the choice of stuffer DNA is important; normally human intronic sequences are used.

Generation of gutless vectors requires a helper virus, thereby giving the name of HDAd to gutless vectors. Permissive cells, usually Cre/293 cells which express endogeneous Cre recombinase are transduced with a helper adenovirus containing most of the adenoviral genome, but the packaging signal spanned by loxp sites (47). At the same time, an HDAd vector containing the transgene and stuffer sequences, flanked by 3' and 5' ITRs is also transfected. The helper virus encodes all the proteins required to produce the virus, however Cre recombinase produced by the cell cuts at the loxP site, which removes the packaging signal and prevents the helper virus genome from being packaged. On the other hand, the HDAd genome containing the packaging signal is packaged, resulting in production of a complete viral vector expressing only the transgene.

After administration to lungs, HDAd vectors exhibit reduced long-term toxicity and prolonged transgene expression compared to first and second generation adenoviral vectors (48, 49). However, the innate immune response remains because it is triggered by the adenovirus capsid proteins. In contrast, the adaptive immune response, which involves T and B cell immunity, is drastically reduced. This is because activation of T cells usually requires viral gene production in cells, which allows for the viral epitopes to be presented in complex with MHC and HDAd vectors completely lack any viral gene expression unlike the parental adenovirus. Also, development of B cell or humoral immunity results in antibody secretion, but B cells usually depend on T cell for activation and in absence of T cell help, there is automatically reduced B cell activation (45).

Many mammalian viruses encode proteins or decoy RNAs that can inhibit the activity of protein kinases (PKR). For instance, adenovirus encodes 160 nucleotide long RNA transcripts referred to as viral-associated (VA) RNAs, VA RNAI and VA RNAII. These VA RNAs are produced as RNA polymerase III transcripts (50). Similarly, plant viruses have evolved strategies to suppress RNA silencing which enhances viral replication and pathogenesis in plants. Recently, the concept of mammalian viruses encoding suppressors of RNA interference has emerged in the context of adenoviruses as well as HIV (51). Studies have demonstrated that VA RNA can act as inhibitors of RNAi in mammalian cells. VA RNAI is expressed at very high levels in adenovirus infected cells and potently inhibits RNAi induced by short hairpin RNAs along with human microRNA precursors (50). Along with VA RNAI, VA RNAII has also been shown to inhibit RNAi. This process involves processing of VA RNAs by Dicer to generate active small interfering RNAs, which are then incorporated into the RISC complex (52). The process of inhibition involves VA RNA acting as competitive substrate which saturates the Dicer and RISC machinery, thereby inhibiting other RNAi processing within the cell. The FGAd vectors are expected to produce VA RNAs and hence delivery of siRNA using FGAd vectors may not be that efficient due to the ability of VA RNAs to suppress RNAi in cells. In contrast, HDAd vectors do not encode any viral genes, hence there are no VA RNAs produced, due to which no silencing of siRNA is observed using HDAd vectors.

Airway gene delivery

In 1997, our group developed a transgene cassette based on K18 regulatory elements for tissue specific transgene expression (53). Cytokeratin K18 is a Type I filament protein (54) and its expression is restricted to single layered epithelial cells (55) including intestine, lung, breast, uterus and liver. We constructed various constructs derived from human K18 gene containing minimal promoter along with enhancer elements, first intron and 5'-UTR. SEAP was used as a reporter gene and combination of the above parts were used to drive reporter gene expression. Highest expression was achieved with the construct containing promoter, enhancer and first intron. The construct was also tested *in vitro* by using lacZ as a reporter (HDK18LacZ) and results showed epithelium specific expression of the transgene. This study provided the proof of principle that human K18 regulatory elements can be used to design gene therapy vectors with epithelium specificity. Tissue specificity of gene expression using K18 promoter was also tested by generating transgenic mice harbouring K18LacZ construct (56). Adult lung showed homogeneous lacZ expression along the epithelial lining of the respiratory system, in conducting airways stopped at the end of cuboidal epithelial and beginning of squamous epithelial cells. Gene expression was also observed in submucosal glands, which was assessed by periodic-acid Schiff staining which stains mucopoly-saccharides. However, staining was restricted to epithelial cells with no expression in other tissues and the localization of lacZ expression correlated well with K18 expression in humans. At the same time the pattern of lacZ expression also resembled the expression of human CFTR which is the target of CF gene therapy. In contrast to FGCMV (first generation cytomegalovirus) vectors, which transduce the entire lung epithelium (Figure 1B), HDAdK18 only transduce the epithelial cells within the airways (Figure 1A). Although lacZ

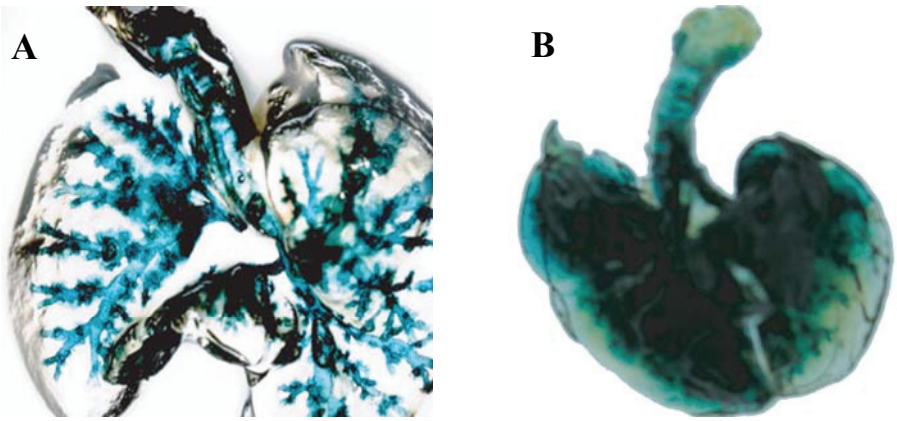

Figure 1. Helper-dependent adenoviral vectors with K18 construct specifically target the airway epithelium with efficiency levels comparable to FGCMV vectors. (A) Delivery of HDK18LacZ vector to the mouse lung. (B) Delivery of FGCMVLacZ vector to the mouse lung. Vector delivery and subsequent lung staining were performed as described previously (59).

expression using both vectors is similar at short time points, after 28 days, gene expression is only sustained with the use of HDAd vectors (49). Therefore, HDK18 vectors provided a tool for directing sustained transgene expression specifically to the epithelial cells. This is one of the primary requirements for effective lung gene therapy.

In 2000, our group developed another gene therapy vector based on previous K18 based vectors, with expression levels rivalling that seen with viral promoters such as CMV (57). In this vector, the 3'-SV40 early poly A was replaced with the late poly A, resulting in dramatically increased gene expression. Additionally, a vector containing the 3' UTR of K18 gene containing intron 6, exon 6, exon 7 and poly A and 3' flanking sequences was then tested for expression. The addition of intron 6, exon 7 and poly A signal at the 3' end of the vector also improved gene expression to levels better than observed with CMV-based vectors.

The K18 based cassette was used to generate an inducible CFTR expression system in IB3 cells as an *in vitro* model for CF gene therapy (58). IB3 contain the CFTR mutation delta F508 mutants, thus mimicking the CFTR defect seen in CF patients. The K18 based cassette with CFTR was used to drive CFTR expression from a Tetracycline (Tet) inducible promoter. In this vector, tetracycline or derivatives of tetracycline (such as doxycycline) would turn on CFTR expression from the Tet inducible promoter, while at the same time, K18 would limit expression to epithelial cells, providing a second stage of tight regulation. This vector system was used to correct the CFTR defect in IB3 cells. The correction of CFTR channel was analyzed using iodide reflux assays. The tetracycline controlled transactivator (rtTA) system was also tested in mice using lacZ as a prototype gene; the results confirmed the tissue specificity and Tet inducibility as seen in IB3 cells. This system could be extended to control the levels of CFTR expression in the context of gene therapy and hence, provided another level of control on gene therapy vectors. K18 based HDAd vectors were also tested for gene delivery in CFTR knockout mice (59) and upon delivery the vector expression was localized to airway epithelia; gene expression could be detected up to a period of 28 days at both the mRNA and protein level. In order to assess the therapeutic benefit of vector delivery, mice were challenged with a clinical strain of *Bulkholderia cepacia complex*. CFTR knockout mice without any gene therapy developed severe histopathology of the lung with high bacterial count. In contrast, CFTR knockout mice receiving gene therapy showed significantly reduced lung histopathology and the levels of bacteria were also significantly reduced to that seen in wild type mice, indicating that gene therapy could help CF patients by reducing susceptibility to opportunistic infection. We also tested the efficacy of using HDK18 constructs in rabbits. Our results demonstrated efficient transduction of a variety of airway epithelial cells. Approximately 66% of tracheal cells were efficiently transduced with virus in 0.1% LPC, an agent which opens tight junctions (60). Figure 2 clearly illustrates the efficient delivery and airway epithelium-specific gene expression obtained by using HDK18LacZ vectors in rabbits.

siRNA delivery

Traditionally, delivery of siRNA has been mediated by plasmids; however, plasmid delivery is extremely inefficient, especially to the lung. Therefore, viral vectors have been generated and evaluated for their ability to deliver shRNA (short hairping like RNA) to the lung. Several groups reporting used either lentiviral systems or first generation adenoviral vectors to test siRNAs. However, since first generation adenoviral vectors can induce a potent inflammatory response, they are not good candidates for delivery of anti-inflammatory siRNA (see above). The development of HDAd with significantly reduced immunogenicity has led to their use as vectors for delivery of anti-inflammatory siRNAs. Additionally, since HDAd vectors do not express any VA RNAs, they do not affect the siRNA processing ability of the cell, in contrast to what may be a potential problem with FGAd vectors.

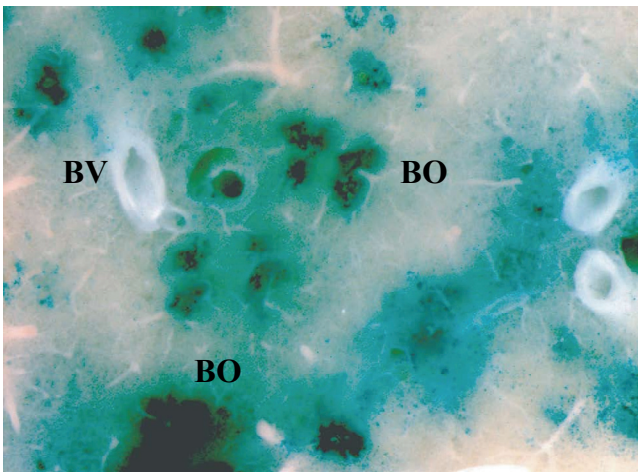

Figure 2. **Specific targeting of the airway epithelium upon gene delivery in rabbits.** Intracheal aerosol administration of HDK18LacZ vectors was performed in rabbits and X-gal staining was performed on the whole lung, followed by histological analysis of the lung as described previously (BO, Bronchioles; BV, Blood Vessels) (60).

Our group has developed an expression cassette under the control of U6 promoter to deliver shRNA; its efficacy has been demonstrated in its ability to target IL-8 mRNA (61). Because of the advantages of adenoviral vectors both in transfer efficiency and episomal stability, HDAd vectors were used to deliver shRNA constructs. Two cell lines were analyzed: IB3 lung epithelial cell line with defective CFTR and C38 cells with corrected CFTR. Treatment of the cells lines with proinflammatory cytokines TNF-α and IL-1β resulted in significant upregulation of IL-8 expression. This response could be downregulated by 70-80% using our U6shRNA constructs. Similar levels of IL-8 downregulation could be observed in cells treated with heat-inactivated bacteria to induce IL-8 expression, by using our siRNA construct. The down-regulation of IL-8 by our vector was gene specific, because vector treatment of cells did not affect levels of NF-κB induction or other cytokine levels such as those of IL-6. Therefore, our siRNA cassette system using HDAd can be effectively used to downregulate inflammatory cytokines in a gene-specific fashion.

Potential challenges

Innate and adaptive immune responses pose the biggest challenge to lung gene therapy. Epidemiological studies indicate there is significant prevalence of the neutralizing antibody anti-Ad5. The frequency of occurrence ranges from approximately 37% of the population in the USA to 85% of the population in South Africa (62). Neutralizing antibodies reduce the efficiency of vector uptake by binding to the vector particles and preventing their uptake in cells (63).

Additionally, the innate immune response which is comprised of natural killer (NK) cells, macrophages, neutrophils, complement as well as many cytokines, is the first barrier encountered by viral vectors. Adenoviruses can infect vast kinds of cells including the macrophages, that are also responsible for uptake of neutralized viral particles (64). Studies have indicated that macrophage depletion can account for up to 95% reduction in levels of inflammatory cytokines in response to inflammatory stimuli (65). Therefore, macrophage depletion has been used in adenoviral gene therapy. This approach has resulted in a significant enhancement of the transduction efficiency of adenoviral vectors as well as a decrease in vector-directed immune response (66). With significant improvement in vector design and development of HDAd, the immune responses can be reduced further. Additionally, to further reduce the capacity of vector to infect macrophages and dendritic cells, we expect that modification of the viral capsid with poly-ethylene-glycol (PEG) can be beneficial in a further reduction of inflammatory response (67). At the same time, non-specific transduction can result in expression of delivered genes in antigen presenting cells, which can directly prime the immune response. In this way, our HDK18 system provides an additional benefit because gene expression is restricted to airway epithelium. In spite of these advantages, one of the drawbacks of the HDAd system is that it requires vector readministration. Our studies have indicated that although first round of vector administration does not induce an immunological response, subsequent rounds of readministration, result in a gradual decrease in transgene expression and an increase in anti-Ad neutralizing antibody titer (68).

The host immune response is one obstacle to airway gene therapy. A second obstacle is the physical barrier to airway gene therapy. Physical barriers to lung gene therapy include surfactant proteins and mucus (69). In addition, mucociliary clearance system, glycocalyceal barrier in the airways and slow rate of luminal endocytosis by epithelial cells, all decrease the efficiency of gene transfer (70). The lumen of the human airways is lined with mucociliary epithelium which is comprised of ciliated cells along with mucus secreting goblet cells. At the same time, in chronic diseases such as cystic fibrosis the submucosal glands enlarge, there is as increase in the number of mucus secreting goblet cells and these cells also appear in distal airways where they are normally absent (71). In this setting, there is a significant increase in levels of mucus production along with impairment of mucociliary clearance, which eventually results in chronic bacterial infection. The build-up of bacterial biofilms along with chronic inflammation further increases the viscosity of the mucus in the airways and acts as a significant barrier against gene therapy vectors. Treatment with mucolytic agents such as nacystelyn or *n*-acetyl-cysteine with cationic liposomes mediated gene transfer has been shown to result in increased transduction efficiency (72). Additionally, primary human airway epithelial cells expressing mucins such as MUC1 are eight fold less refractory to adenoviral mediated transduction compared to cells not expressing mucin (73). Even after deletion of MUC1 in mice, adenoviral transduction was very inefficient, due to other components of glycocalyx (74).

Airway epithelial cells also secrete other matrix components such as collagen and fibronectin, which further limit the ability of gene therapy vectors (75).

Concluding remarks

There has been tremendous development in the field of adenoviral gene therapy over the last decade from the initial use of first generation adenoviral vectors to the recent development of helper-dependent or so called gutless viral vectors. The gutless vectors have demonstrated significant improvement over first generation vectors in terms of toxicity and efficiency. Using cell specific promoters such as cytokeratin 18, genes can be targeted specifically to the airway epithelium, which limits a general inflammatory response. As a result of reduced toxicity, HDAd vectors can also be used for delivery of anti-inflammatory siRNAs, which was a serious problem in FGAd vectors. Although, HDAd vectors resolve many critical problems in pulmonary gene therapy, there are still a number of hurdles yet to overcome. It is hoped that future work will be able to address problems associated with the innate immune responses and the problems generated by readministration. We have only cleared the first two hurdles that will take us from laboratory bench to general clinical use.

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