Physical and Biological Barriers to Viral Vector–mediated Delivery of Genes to the Airway Epithelium

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A gene therapy for cystic fibrosis (CF) lung disease by intralumenal delivery of therapeutic transgenes into the lung is a logical treatment strategy if efficient gene transfer can be achieved without detrimental effects to the patient. Indeed, pioneering work in the late 1980s showed that genetically engineered viruses could deliver the CF corrective transgene to cultured cells from patients with CF. However, after many attempts to deliver the corrective gene to the lungs of patients with CF *in vivo* and with the luxury of 20/20 hindsight, it is realized that although logical, the strategy to accomplish this task did not appreciate the evolution of the lung to resist invasion by pathogens such as viruses. It is now apparent that several levels of barriers exist that restrict exogenous gene delivery to the airway epithelium by commonly used viral vectors. Components of the innate and cell-mediated immune system collectively limit both the access to and duration of gene transfer vectors to the airway epithelium. Alternative viral vectors that have evolved to circumvent these barriers will require further development if gene transfer is ever to be considered a therapy for CF lung disease.

Keywords: cystic fibrosis; gene therapy; viral vectors

In this short article, I will sum up experiences of my laboratory using adenoviral vectors (AdV) as gene delivery vehicles for the respiratory epithelium, the target tissue for the treatment of the underlying cause of cystic fibrosis (CF) lung disease. This work has mainly focused on the "innate" physical and biological barriers posed by the airway epithelium that limit gene transfer efficiency, with the opinion that until efficient gene transfer to the correct target cells can be achieved the prospect for a safe CF gene transfer strategy will be limited. This review will discuss mainly my conclusions from work performed in my laboratory in the context of work performed by others in the field. Additional barriers to effective and safe gene transfer such as the host cellular immune response against vectors and/or transgene antigens and the mechanisms of delivering gene transfer vectors to the lung will not be discussed here, and the reader is referred to several recent reviews that address these issues (1, 2).

Several separate scientific advances culminated in the idea that a gene transfer approach for CF lung disease would be feasible. Many years of basic research with a common lung virus, the adenovirus, had generated less pathogenic replication-deficient vectors capable of expressing a transgene of choice. Secondly, in 1989, the defective gene that results in CF disease was cloned and the normal product of this gene identified as a cAMP-activated chloride ion channel, the cystic fibrosis transmembrane conductance regulator (CFTR) (3).

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The first signs of CF lung disease occur in the distal bronchiolar airways. With time, bronchiolitis and mucus plugging of the small airways are common findings in the CF lung. The link between CF pathogenesis and the CFTR defect is related to the normal function of CFTR maintaining hydration of the periciliary fluid layer that lines the airway surface (4). An absence or expression of defective CFTR in the lung results in reduced chloride ion secretion, hyperabsorption of sodium ions, increased viscosity of airway secretions, impaired mucociliary clearance, chronic bacterial infection, bronchiectasis, and premature death (5). Because these pulmonary manifestations are likely primary or secondary to loss of CFTR function, the most efficacious strategy to treat CF lung disease would be to replace normal CFTR function in airway epithelial cells, thus "correcting" lung epithelium homeostasis and hopefully lung function.

The airway epithelial cell types that exhibit all of the ion- and fluid-transporting functions of CFTR and display abnormal function in patients with CF are the ciliated epithelial cells (6), which are considered to be the target cell types that require correction (7, 8). However, immunolocalization studies have identified the serous cells of the submucosal glands as the highest CFTR-expressing cell type in the lung, suggesting that these cells may also be an important target for gene replacement (9).

Shortly after the identification of the CFTR gene, two groundbreaking observations made gene therapy for CF lung disease appear imminent. First, isolated epithelial cells derived from the airway epithelium of patients with CF and cultured on plastic dishes could be phenotypically "corrected" by transferring the CFTR cDNA into the cells (10). Secondly, replication-defective AdV engineered to express the CFTR cDNA were administered to the airways of experimental animals, and transgene expression was observed in respiratory epithelium (11). These pioneering studies produced a flurry of scientific activity and excitement in both the gene therapy and CF scientific communities, and within 3 years of these initial observations the first clinical trial describing AdV-mediated gene transfer to the airway epithelium of patients with CF *in vivo* was reported (12).

Over a decade later, these promising early observations have unfortunately not withstood further investigation. After over 20 gene therapy clinical trials for CF lung disease (of which greater than 70% used AdV), the gene therapy community has realized that gene transfer to airway epithelium *in vivo*, although logical, is not trivial. The evolution of the respiratory epithelium as an effective barrier to invading pathogens entering the lung (e.g., viruses), by a host of innate and cell-mediated immune systems, culminates in restricted cellular uptake of gene transfer vectors and reduced transgene expression.

VIRAL VECTORS AS GENE DELIVERY VEHICLES TO THE LUNG: AMBITIOUS OR FAR-SIGHTED?

For most viral vectors proposed over the last 10 years for CFTR delivery to the lung, the targeting capacity and efficiency of gene transfer have been rate-limiting to the success of this approach. The gene transfer efficiency required for physiological correction

of CF lung disease was first tested by Johnson and colleagues, who showed in CF cell–mixing experiments that 6 to 10% of CFTR-expressing cells were required to restore normal levels of chloride secretory function to an epithelium *in vitro* (13). However, this degree of "correction" was insufficient to correct the hyperabsorption of sodium ions that is likely to be necessary for resolving CF lung disease. To restore the normal sodium transporting capabilities of the respiratory epithelium, it has been hypothesized (but not proven) that greater than 80% of the surface epithelial cells will have to express CFTR (7). Therefore, the number of CF cells to be targeted by a vector, i.e., ciliated cells, required to express CFTR will be high. Even so, it must be emphasized that correction of the chloride secretory defect by replacement of functional CFTR has not yet been shown to restore ionic homeostasis to the CF lung or reverse CF-related pathology.

The safety of gene transfer to airway epithelium *in vivo* using viral and nonviral vectors in clinical trials has so far been promising. However, determination of the efficiencies of gene transfer in the trials performed to date have shown, at best, only partial "correction" ($< 20\%$) of the CF bioelectrical defect (12, 14–16). No attempts to determine efficacy of gene transfer on CF lung disease-related end-points have yet been attempted.

IDENTIFYING AND APPRECIATING THE TARGET CELL TYPES FOR CFTR DELIVERY *IN VIVO*

Adenoviral vectors are extremely efficient gene delivery vehicles once the virus has entered into the target cell. Why then is this respiratory virus apparently so inefficient at targeting the intact epithelium *in vivo*? One consideration when comparing wild-type adenovirus infection to transduction by AdV is that the replication-deficient vectors rely on delivering many virus particles to a target tissue, whereas wild-type virus only requires access to a small number of cells from which it can propagate and spread within the target tissue. Replication-competent wild-type Ad may take advantage of regional differences in airway epithelium integrity and injured epithelium has been shown to be more susceptible to AdV transduction than intact epithelium (17).

Another misconception about AdV infection of cells was the correlation of gene transfer efficiency to isolated airway epithelial cells *in vitro* to that expected *in vivo*. The failure of the lung gene transfer community to appreciate the complex phenotypic and morphologic characteristics of the target airway epithelial cells *in vivo* resulted in trying to "force-feed" viruses into the target cells without sufficient attempts to understand the mechanisms that were restricting viral entry into the cell.

Airway epithelial cells are present throughout the conducting airways of the lung, including the nasal, tracheal, bronchial, and bronchiolar regions. Airway epithelial cell type composition is complex because it is species- and airway region–dependent, and the reader is referred to comprehensive reviews describing species-specific airway epithelial cell distribution (18, 19). For the human lung, the conducting airways are composed of several epithelial cell types dependent on airway region. Generally, the surface epithelium is composed of ciliated cells, mucus-secreting cells (goblet), serous cells, Clara cells, and basal cells. Alveolar regions of the lung consist primarily of alveolar Type I and Type II cells, although these cell types are not thought to participate in the pathophysiology of CF lung disease and hence are not considered targets for CFTR gene transfer.

The lumen of human cartilaginous airways (nasal, tracheal, and bronchial) is normally lined with a pseudostratified mucociliary epithelium comprised of ciliated cells and mucus-secreting goblet cells overlying basal epithelial cells (Figure 1). A subsubpopulation of basal epithelial cells are considered to be the stem cell precursors for the ciliated and mucus cell phenotypes (20). The morphology of the human lower airway epithelium is distinctly different from the upper airway epithelium, with the lumen of the bronchiolar regions normally lined with simple cubiodal ciliated epithelium with few mucus-secreting cells and no basal cells. Because ciliated epithelial cells are terminally differentiated and basal epithelial cells are absent from the bronchiolar regions, it has been speculated that mucus-producing cells (Clara cells) may be the progenitors of ciliated cells in these regions. Overall, it is considered that the ciliated cells in both upper and lower airway regions are the target cell types for CFTR gene delivery.

GENE TRANSFER EFFICIENCY TO AIRWAY EPITHELIUM IS LOW DUE TO CELLULAR BARRIERS TO VIRAL ENTRY

Unlike for isolated airway epithelial cells *in vitro*, it is now apparent that there are several extracellular barriers to AdV on the human airway lumenal surface that result in inefficiency of gene transfer *in vivo* (Figure 1). They include the mucociliary clearance system, the glycocalyceal barrier, the absence of the adenoviral receptors from the airway lumen, and the slow rate of lumenal endocytosis of airway epithelial cells (21–23).

A difficulty in the field of CF lung gene transfer has been identifying a model that recapitulates the phenotypic and morphologic characteristics of the human lung airway epithelium *in vivo*. Although mouse models are attractive because they are inexpensive and can be easily manipulated genetically, the airway epithelial cell type distribution in the murine airway (with the exception of the murine nasal epithelium) is not reflective of the human. A significant advance in the development of airway models was the generation of *in vitro* cell culture models derived from human primary airway epithelial cells obtained from patients with or without CF undergoing lung transplantation (22). Isolated airway epithelial cells grown over a period of 1 to 2 months at an air–liquid interface result in the generation of a pseudostratified, mucociliary airway epithelium that displays similar morphologic and phenotypic characteristics of the *in vivo* human cartilaginous airway epithelium (Figure 1). Recent studies have revealed that this model system recapitulates the phenotypic differences that occur between CF and non-CF airway epithelium: cultures from patients with CF display reduced chloride ion transport, hyperabsorption of sodium ions, the failure to regulate the depth of airway surface liquid, and the dehydration of secreted mucus that results in cilial dysfunction and mucostasis (4). Human airway epithelial cell (HAE) culture models have now been used by a number of different groups to test the usefulness of viral and nonviral vectors for delivering genes to the airway epithelium and have been predictive for gene transfer to human airways *in vivo* (22, 23).

For example, AdV-mediated gene transfer to airway epithelial cells grown on plastic is highly efficient (24), with cellular transduction efficiencies of 90 to 100%, and when the transgene is CFTR, full correction of the spectrum of CF bioelectrical defects is obtained (25). In contrast, inoculation of the apical surface of HAE by AdV does not result in efficient gene transfer, whereas basolateral inoculation is successful at targeting basal epithelial cells (26). However, columnar epithelial cells including ciliated cells are susceptible to AdV infection from the lumenal surface if HAE are inoculated after transient disruption of the epithelial tight junctions (27) (*see* below and Figure 2). These data suggest that the apical surface of polarized respiratory epithelium is restrictive to AdV penetration. These observations confirmed the earlier data derived from *in vivo* epithelial cell models derived from upper airway regions of rodents, nonhuman primates, and humans that showed that: (*1*) transgenes were

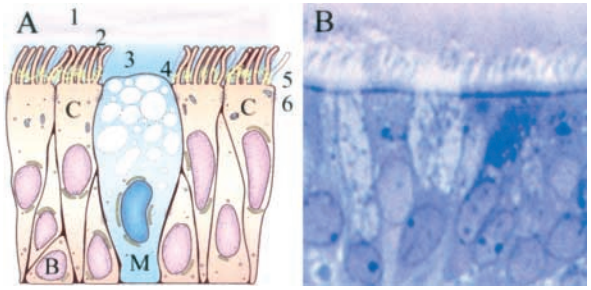

Figure 1. Cell type composition of the airway surface epithelium and potential barriers to gene transfer vectors. (*A*) The airway epithelium is composed of ciliated cells (C), mucus-containing cells (M) overlying basal epithelial cells (B). Gene transfer vectors delivered via the intraluminal route encounter several barriers that may limit gene delivery to target cells in the airway epithelium: (*1*) secreted mucus; (*2*) structure and motion of the cilia; (*3*) an airway surface fluid; (*4*) a robust and structurally diverse glycocalyx; (*5*) an apical membrane that has a limited endocytotic capacity; and (*6*) epithelial tight junctions that restrict access to basolateral membranes. (*B*) Histological cross-section of a human airway epithelial cell culture grown at the air–liquid interface showing ciliated, mucus and basal cell composition. This *in vitro* model of the human airway epithelium recapitulates the physical and biological barriers to gene transfer vectors as seen for the airway epithelium *in vivo*. *Original magnification*: ×100 (Richardson's Stain).

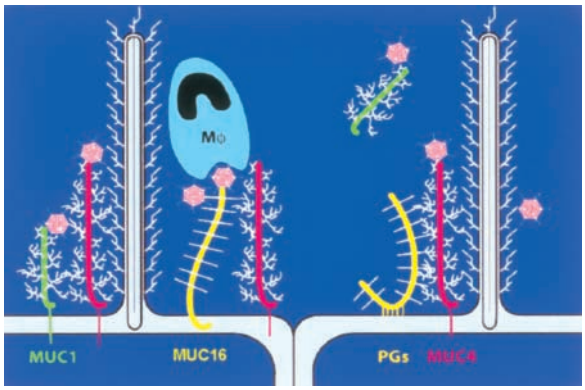

Figure 3. Restriction of AdV access to the apical membrane of airway epithelium by the highly glycosylated and abundant glycocalyx. Schematic of the highly glycosylated molecules on the human airway lumenal surface that comprise the glycocalyx layer. Glycoconjugates such as tethered mucins (MUC1, MUC4, MUC16) and proteoglycans (PGs) cover the microvilli-rich airway surface and may attach to and restrict the access of AdV to the apical membrane. The fate of AdV attached to these structures may be engulfment by airway macrophages (MΦ) and/or incorporation into the secreted mucus after shedding of the glycoconjugate-AdV complex from the apical surface.

expressed after *in vivo* dosing in less than 20% of the surface epithelial cells; and (*2*) that basal epithelial cells were efficiently transduced by AdV (17, 24). Subsequently, it was determined that although both columnar and basal epithelial cells express the receptors required for AdV entry into cells, the human coxsackie B and adenovirus 2 and 5 receptor (hCAR, [28]) and, $\alpha_v\beta_{3/5}$ integrins (29), expression in columnar cells is restricted to the basolateral domain of the cell membranes (22, 23). In fact, hCAR has been localized to regions associated with epithelial cell tight junctions (30), a location that may restrict access of AdV to hCAR delivered by apical and/or basolateral routes. This observation may explain why in our hands basolateral inoculation of HAE by AdV failed to efficiently transduce columnar cell types (Figure 2).

THE GLYCOCALYX AS A RESTRICTION BARRIER FOR ADV DELIVERY TO THE AIRWAY EPITHELIUM

Because the receptors for AdV cellular entry are localized to the basolateral compartments of the epithelium and retargeting strategies are dependent of identification of suitable surrogate receptors, we investigated whether redistribution of hCAR to the apical surface of polarized cells would improve the efficiency of AdV-mediated gene transfer. To achieve this, we expressed the external domain of hCAR (containing the AdV attachment site) at the apical surface of polarized epithelia by incorporation

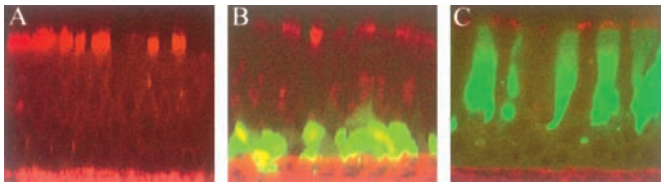

Figure 2. Polarized susceptibility of HAE cultures to AdV infection. Confocal XZ optical sections of HAE cultures inoculated with AdVGFP and 48 hours later, GFP (*green*) expression assessed: (*A*) Inoculation of the apical surface of HAE results in low gene transfer efficiency. (*B*) Inoculation of the basolateral surface results only in efficient gene transfer to basal epithelial cells. (C) Efficient gene transfer to columnar cells after AdV inoculation of the apical surface immediately after tight junctional disruption by sodium caprate. Cilia at the apical surface are identified with anti–β-tubulin conjugated to Texas Red (*red*). *Original magnification*: ×63. Figure reproduced with permission from the *Journal of Virology* (26).

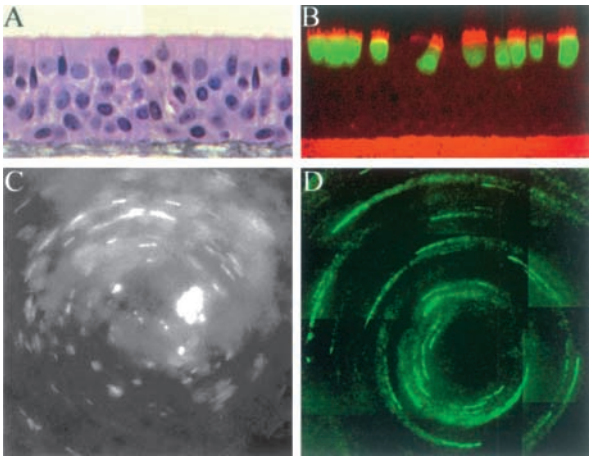

Figure 4. Ciliated cells are infected by RSV and release progeny exclusively from the apical surface. (*A*) Cross-section of HAE culture demonstrating the morphology of the cells at the time of inoculation of the apical surface by RSV-GFP. (*B*) Forty-eight hours later, GFP expression (*green*) was seen exclusively in ciliated columnar cells identified by a cilial specific marker (*red*). (C) Long-exposure time image of *en face* view of an HAE culture with fluorescent beads incorporated into the mucus layer showing circular directionality of the mucus layer produced by coordinated cilial beat. (*D*) *En face* view of an HAE culture infected with low titer RSV-GFP and 48 hours later, GFP expression assessed. Circular spread of RSV-GFP infection mirrors that of mucus movement on the apical surface, suggesting that spread of RSV is facilitated by the coordinated cilial beat. *Original magnification*: *A* and *B*, ×63; *C* and *D*, ×5. Reproduced in part with permission of the *Journal of Virology* (26).

of a glycosylphosphatidylinositol-linker (gpi-hCAR), and found that although this chimeric receptor was expressed at the apical membrane, gene transfer efficiency with AdV still remained low. These studies ultimately identified the lumenal surface glycocalyx as another barrier to AdV that functions as a "barbed-wire fence" to protect the apical surface including apical receptors from lumenal insult (21). We have now confirmed this barrier function of the airway glycocalyx to AdV with HAE cultures and mouse tracheal epithelium *in vivo* expressing gpi-hCAR (31). However, the extent of the barrier effect of human airway glycocalyx in restricting AdV access remains controversial (32).

The glycocalyx on the airway epithelium lumenal surface is composed of several families of carbohydrate-rich molecules, including glycoproteins (most notably the mucins), proteoglycans, and glycolipids. A major component of the airway glycocalyx are the "tethered" mucins, particularly the large (> 1 megadalton), heavily glycosylated MUC1 and MUC4 glycoproteins (33, 34). With respect to airway gene transfer, sialoglycoconjugates (including MUC1) expressed on the apical surface of polarized epithelial cells inhibit AdV-mediated gene transfer (35). Several mucin species will also be present in the mucus in the airway lumen and may act as false attachment sites for AdV thus effectively reducing the amount of AdV that ultimately reaches the apical surface receptors required for viral penetration (Figure 3).

The fate of AdV bound to airway glycocalyx components is speculative. One possibility is that mucins may passively or actively "present" the bound AdV for recognition and engulfment by resident airway macrophages. It has been shown in the murine airway that early after AdV administration to the lung, macrophages migrate to and internalize AdV that are present in the airway lumen possibly attached to glycocalyx structures (36). It has been estimated that 70 to 90% of AdV are sequestered by airway macrophages 24 hours after intraluminal administration to the lung (37). We predict that glycocalyx components present AdV to incoming macrophages for phagocytosis and degradation. In addition, tethered mucins are shed from the airway surface and incorporate into the soluble mucin layers (34). This property of airway mucins raises the possibility that AdV attached to shed tethered mucins may be eliminated from airways by incorporation into the mucociliary transport system.

OVERCOMING THE LIMITATIONS OF LOW EFFICIENCY OF AdV-MEDIATED GENE TRANSFER

To date, two main strategies to improve the efficiency of gene transfer of AdV after intralumenal delivery have been attempted. One approach is to retarget AdV to nonviral receptors present on the apical surface of lumenal epithelial cells. The other strategy attempts to access the basolateral surfaces of the epithelial cells either by nonlumenal delivery of vector or by disruption of epithelial "tight" junctions.

Retargeting AdV to Apical Receptors to Increase Gene Transfer Efficiency

Retargeted AdV can successfully transduce cell types that are usually refractory to AdV infection due to lack of attachment/entry receptors. The epidermal growth factor receptor, stem cell factor receptor, fibroblast growth factor receptor, α_V integrins, and T cell receptors (CD 3) have all been used as surrogate receptors for AdV entry in a variety of cell types (38–40).

Retargeting AdV to receptors on the airway lumen required identification of suitable receptors on the target ciliated cells that are conducive for AdV attachment and entry. Examples of such receptors are members of the 7-transmembrane spanning G protein–coupled receptor family (i.e., P2Y2 purinoceptors, B2-kinin receptors, and adenosine type 2b receptors). These receptors were identified as putative utile target receptors for redirecting AdV tropism to the surface epithelium of the lung because they are highly expressed on the luminal surface and are internalized into cells upon activation (41). Other receptors proposed as targets for redirecting gene transfer vectors are the urokinase plasminogen activator receptor and the SEC-2 receptor (42, 43).

Retargeting of AdV has so far been achieved by chemically, immunologically, or genetically modifying the AdV capsid coat by incorporating new receptor ligands that can target candidate receptors. As a "proof of concept" study, an hemagglutin (HA)-epitope tagged P2Y2 receptor expressed at the apical surface of HAE was targeted with bi-specific antibodies consisting of antibodies to AdV fiber-knob protein/HA-tag and in combination with glycocalyx abrogation shown to facilitate AdV entry into columnar cell-types (44). Similar retargeting has been shown with chemical conjugation of AdV to receptors via a biotin–streptavidin "bridge" (41).

Retargeted AdV with fiber-knob protein modified to express novel ligands that can interact with target receptors have been developed, and the feasibility of this approach has now been reported by a number of groups (39, 45).

Strategies to Target Endogenous AdV Receptors

The localization of viral uptake pathways to the basolateral surfaces of airway epithelial cells suggests that delivery to this surface could be beneficial for improving gene transfer. Such an approach may also allow targeting of basal epithelial cell subpopulations that may function as stem cells for the columnar epithelium resulting in gene transfer to the lung for the lifetime of the individual. Targeting stem cells is an important consideration for gene transfer to the airway epithelium because ciliated cells have a relatively short lifetime (40–90 days), suggesting that strategies to directly target ciliated cells will require readministration of vectors every 1 to 3 months.

Access to basal cells/basolateral surfaces after intravenous administration of vectors requires vector dissemination through the lung blood vessel wall and the surrounding connective tissue, as well as penetration of the basal lamina underlying the respiratory epithelium. Sufficient access to airway epithelial cells via this route has not yet been demonstrated (46).

An alternative approach to target endogenous AdV receptors on columnar cells is transient disruption of epithelial tight junctions, thus exposing hCAR, before inoculation by AdV. Walters and coworkers have shown that treatment of the apical surface of HAE with the calcium chelator EGTA or hypotonic solutions (e.g., water) allows for improved AdV-mediated gene transfer (47). The short-chain fatty acid, sodium caprate, has also been shown to increase AdV-mediated gene transfer to HAE and mouse tracheal epithelium *in vivo* (27, 48). The ability for tight junctional modulation to potentially allow for AdV access to basal epithelial stem cells remains to be investigated.

However, even with suitable retargeting strategies for AdV the requirement of a vector to circumvent the glycocalyx barrier remains. It is unlikely that effective and safe strategies to abrogate the airway glycocalyx sufficient for gene delivery improvement can be developed. Whether further modifications to the AdV capsid that result in less affinity of the virus for glycocalyx structures could be achieved has not yet been investigated.

IDENTIFICATION OF OTHER VECTORS THAT CIRCUMVENT THE PHYSICAL BARRIERS TO GENE DELIVERY

The focus of this review has been on the use of AdV for use in treatment strategies for CF lung disease. A number of other

vectors have been suggested as candidates for CF lung gene transfer vectors and have been rigorously investigated. Adeno-associated viruses, retroviruses, lentiviruses, and liposomal vectors have all shown promise in preclinical studies in the lung, and some have been tested in clinical trials. Although most of these vectors can be delivered to the human airway epithelium relatively safely, the efficiency of gene transfer achieved by these vectors has been low, suggesting that barriers to viral entry into the target cell types may exist. Strategies to improve gene transfer efficiency for these other vectors have paralleled the strategies for improving AdV gene transfer. Whether efficiency can be improved sufficiently to shows efficacy of gene transfer in the lungs of patients with CF remains to be determined.

Novel vector types are now being assessed for airway gene transfer that may be more efficient at breaching airway epithelial cell barriers. For example, Sendai virus (SeV), the murine equivalent of human parainfluenza virus type 1, has been shown to infect ciliated and nonciliated cells of rodent airways after intraluminal delivery (49). Human coronavirus 229E has been found to infect human polarized airway epithelia from the apical surface (50), although less than 10% of the cells were infected and the large size of this viral genome complicates its use as a vector. HIV-based lentiviral vectors pseudotyped with Ebola virus envelope proteins have been shown to efficiently transduce airway epithelial cells *in vitro* and murine airways *in vivo* (51), suggesting that combining the efficiency of Ebola virus entry with the potential longer duration of lentivirus-mediated gene expression may provide a useful vector for lung gene transfer strategies. In a similar study, a simian lentiviral vector was pseudotyped with Sendai virus envelope proteins F and HN, which transduced rat polarized tracheal epithelial cells *in vitro* from the apical surface, albeit with low efficiency (52).

We have recently focused on the paramxyovirus (PV) family of human respiratory viruses. These viruses are a family of enveloped viruses with nonsegmented negative strand RNA genomes. Human respiratory syncytial virus (RSV) and human parainfluenza virus (PIV) are, respectively, the first and second leading causes of viral respiratory disease in infants and children requiring hospitalization (53). Although most infections are restricted to the upper airways and resolve within 1 to 2 weeks without treatment, the entire respiratory tract can be infected, resulting in bronchitis, bronchiolitis, and/or bronchopneumonia, especially in immunocompromised patients. Almost everyone has been infected by PV by 2 years of age, but the immunity induced is typically incomplete and reinfection by the same virus is common although subsequent infections are partially restricted and the disease severity reduced (53). With regard to potential gene transfer vectors, it is advantageous that immunity against PV is incomplete and wanes with time.

The tropism of PV for human respiratory epithelium led us to test whether RSV efficiently infected HAE and whether any cell type–specific targeting occurred. The reverse genetics has enabled the rescue of fully recombinant RSV thus allowing for viral genome manipulation and insertion of marker transgenes (54). HAE inoculated with recombinant RSV expressing GFP showed that RSV efficiently infected columnar epithelial cells from the apical surface and exclusively infected ciliated epithelial cells (26). The ability of RSV to transfer genes to the ciliated cells of the airway epithelium after lumenal delivery suggests that this virus may provide a new vector system suitable for disorders of the lung epithelium such as CF lung disease.

RSV infection of respiratory epithelium is however cytotoxic and pulmonary disease due to RSV infection is caused by both direct virus-mediated events and the effects of host immune responses. The most commonly described cytotoxic effect of RSV infection of non-polarized cells *in vitro* is giant cell (syncytium) formation, leading to cell death. This effect is likely due to cell membrane expression of RSV glycoproteins that are fusogenic and can interact with neighboring cells to induce cell–cell fusion (55). However, syncytia formation in respiratory epithelium is rarely encountered in pathological specimens from human fatal RSV infection, unless individuals are profoundly immunosuppressed (53). Indeed our experiments with RSV infection of HAE did not result in ciliated cell syncytia formation, an effect likely due trafficking of the fusogenic viral glycoproteins exclusively to the apical surface of ciliated cells where interactions with neighboring cells would be limited. This conclusion was supported by the observation that RSV assembled at and budded exclusively from the apical surface of infected ciliated cells and spread of virus was propagated by the directional flow of airway surface liquid dictated by cilia beat direction (26) (Figure 4). The apical shedding of RSV was not accompanied by gross cytotoxicity and suggests that RSV spread remains within the environment of the lung an observation that may explain why RSV viremia is exceptionally rare. Nevertheless, the cytotoxicity of replication-competent RSV vectors currently limits their usefulness as gene transfer vectors for "proof of concept" studies.

Further study will be required to generate versions of these vectors that could be used to deliver CFTR to the airway epithelium *in vivo*. A wide variety of attenuated viruses have been created and characterized in the development of live attenuated vaccines against PV (56). Attenuation is defined as a reduced ability of the virus to cause disease and most commonly reflects a reduced replication capacity. Replication-attenuated PV vectors would be desirable for a gene transfer vector. The fact that PV vaccine candidates can be safety administered to infants and young children suggests that attenuated PV may be useful for constructing safer gene transfer vectors (57).

Alternatively, pseudotyped vectors generated using glycoproteins from PV (e.g., RSV pseudotyped lentiviral vectors) could provide vectors that target ciliated cells and have the capacity to improve duration of transgene expression. However, any strategy to target ciliated cells with CFTR can only "correct" a CF epithelium for the lifetime of the CFTR-expressing ciliated cells (40–90 days). Therefore, all vectors directed to ciliated cells, even integrating vectors, will ultimately require repeat administration. In the absence of an identified stem cell for ciliated cells and a suitable vector that can target such a stem cell, the repeat dosing of vectors that target ciliated cells that are safe and can be readministered every 2 to 3 months is an acceptable strategy at the present time.

CONCLUSIONS

A gene transfer strategy for the treatment of CF lung disease still struggles to prove itself as a realistic goal, not least because the immature science of *in vivo* gene transfer was referred to at the outset as "gene therapy." The promise of a gene therapy set unrealistic goals for this approach, for as we have discovered, there are multiple barriers to achieving efficacious and safe delivery of genes to the lung, of which only a few have been discussed here. The field of *in vivo* gene transfer has been aided by many different aspects of basic biological and medical research efforts and the eventual realization of a gene therapy for CF lung disease will only take time and a continuation of these efforts.

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