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Perspective

Gene therapy for the treatment of cystic fibrosis should be a “natural”: Cystic fibrosis (CF) is a recessive disease associated with loss of function mutations in the CF transmembrane conductance regulator (CFTR) gene, which has a well-characterized gene product; heterozygotes, as predicted, appear to be phenotypically perfectly normal; the level of expression of CFTR in affected cells generally appears to be low; and the dysfunctional epithelial lining cells in the organ most affected by CF (the lung) are available for direct vector delivery via topical administration (1). However, despite an impressive amount of research in this area, there is little evidence to suggest that an effective gene-transfer approach for the treatment of CF lung disease is imminent. The inability to produce such a therapy reflects in part the learning curve with respect to vector technology and the failure to appreciate the capacity of the airway epithelial cells to defend themselves against the penetration by moieties, including gene-therapy vectors, from the outside world. This Perspective will focus on the issues that impact on moving this field forward. What is the target for CF gene therapy in the lung? Cystic fibrosis affects the conducting airways of the lung and not the alveolar surfaces. The airways in general consist of a “large” airway (bronchial) region that is lined by a pseudostratified columnar superficial [...]

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Perspective

SERIES
on cystic fibrosis

James M. Wilson, Editor

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Gene therapy for the treatment of cystic fibrosis should be a “natural”: Cystic fibrosis (CF) is a recessive disease associated with loss of function mutations in the CF transmembrane conductance regulator (CFTR) gene, which has a well-characterized gene product; heterozygotes, as predicted, appear to be phenotypically perfectly normal; the level of expression of CFTR in affected cells generally appears to be low; and the dysfunctional epithelial lining cells in the organ most affected by CF (the lung) are available for direct vector delivery via topical administration (1). However, despite an impressive amount of research in this area, there is little evidence to suggest that an effective gene-transfer approach for the treatment of CF lung disease is imminent. The inability to produce such a therapy reflects in part the learning curve with respect to vector technology and the failure to appreciate the capacity of the airway epithelial cells to defend themselves against the penetration by moieties, including gene-therapy vectors, from the outside world. This Perspective will focus on the issues that impact on moving this field forward.

What is the target for CF gene therapy in the lung? Cystic fibrosis affects the conducting airways of the lung and not the alveolar surfaces. The airways in general consist of a “large” airway (bronchial) region that is lined by a pseudostratified columnar superficial epithelium and contains numerous submucosal glands, and a “small” airway (bronchiolar) region that is lined by a simple columnar epithelium and is devoid of glands. Central issues for CF gene therapy are which region (large vs. small) and which tissue (superficial epithelium vs. glands) should be targeted.

Obviously, the answer to this question requires knowledge of the pathogenesis of CF lung disease. As reviewed earlier in this Perspective series (2, 3), this is a controversial issue. Although the so-called “isotonic” and “hypotonic” airway surface liquid theories have different predictions on the pathogenesis of CF airways infection, both agree that defects in the superficial epithelium may initiate CF lung disease. However, studies from other model cell culture systems, like Calu-3 cells and cultured gland acini (4), predict that there may be abnormalities in gland volume/compositional (HCO_3^-) regulation in CF that may be more important in the pathogenesis of CF airways infection. This debate can also be viewed in the context of the individual cell types in the airways. Advocates of the importance of the superficial epithelium in CF pathogenesis likely would favor targeting the ciliated cell, which clearly exhibits all of the ion transport functions of CFTR and exhibits abnormal

function in patients with CF (5), whereas advocates of the importance of the submucosal gland would likely favor targeting the submucosal gland serous cells, which may be the highest CFTR-expressing cell type in the lung (6).

In the absence of definitive data from model systems, from an operational point of view probably the best strategy is to examine the sequence of disease in young CF patients and select the target based on those data. Perhaps the most relevant observations are that CF infants typically present clinically with physical and roentgenographic findings of bronchiolitis, exhibit as their first pulmonary function abnormality small airways obstruction, and have evidence from autopsy studies of mucus plugs in small airways. These data suggest that as in other major airway diseases – chronic bronchitis, for example – small airways are the initial and major site of functional disease (airflow obstruction) in the CF lung. Therefore, restoration of function in the superficial epithelium lining small airways should be clinically beneficial. This reasoning does not dismiss expression of abnormal function in proximal CF airways. Indeed, virtually all studies of epithelial dysfunction in the lung have detected differences in this region, but the importance of small airways obstruction in the phenotype of airways disease suggests that selective correction of epithelial defects in the large airways will not be therapeutically useful. Interestingly, virtually all gene-therapy trials to date have delivered vectors via the topical route to the superficial epithelium, but it is not obvious that aerosol delivery strategies have been optimized for small airway deposition. Although deposition is difficult in patients with airways occluded by mucus plugs and infection, it will be important to develop efficient means to deliver vectors via aerosol to small airways.

However, it is possible that it may be important to treat submucosal glands and that we will not be able to devise strategies to effectively dose CF airways. Therefore, it would appear prudent to continue efforts to deliver vectors systemically that could access gland regions as well as the superficial epithelium of occluded airways.

How much gene transfer is enough? A key issue is to distinguish between the concepts of “level of CFTR transduced/cell” and “percent correction,” denoting the fraction (percentage) of CF cells within an epithelial region (area) that are “corrected.” With regard to level of transduced CFTR/cell, based on endogenous CFTR expression data it is likely that the level required for ciliated cells will be very low whereas the level required for

serous cells will be higher (4). With regard to percent correction, initial studies focused on this issue utilized monolayers of immortalized CF epithelial cells comprised of varying percentages of CF cells and CF cells "corrected" with wild-type CFTR (7). These studies showed that approximately 6%–10% of the cells within a monolayer must consist of "corrected" CF cells to restore normal Cl⁻ transport function.

While informative, the study by Johnson *et al.* emphasizes the importance of both knowledge of the pathogenesis of CF lung disease and the fidelity of the model system to the *in vivo* situation to accurately address this issue. For example, the epithelial model system used for these studies comprised a mosaic monolayer epithelium that was highly connected via gap junctions, utilized "corrected" CF cells that expressed rather high levels of CFTR per cell, and focused only on Cl⁻ transport. The "amplification of correction" (*i.e.*, normalization of function with correction of a small percentage of epithelial cells) reported in that study likely reflected the movement of Cl⁻ ions from non-corrected to corrected cells through gap junctions, with Cl⁻ secretion reflecting exit through a "lot" of CFTR in a small number of corrected cells. It is likely that the number of gap junctions per cell in a well-differentiated epithelium *in vivo* is less than in the immortalized airway cells used in this study, and hence the percentage of cells requiring correction to restore normal Cl⁻ transport *in vivo* may well exceed 10%.

The relationship between normalization of function and percentage of corrected cells within the epithelium is also quite different if one considers Na⁺ transport. Recent data have suggested that lack of CFTR regulation of Na⁺ transport rates may be important in the pathogenesis of CF lung disease and that the relationship between CFTR and Na⁺ transport is more "local," *i.e.*, may involve protein–protein interactions confined to single cells (1). Thus, when abnormal CF Na⁺ transport is used as an index of correction, one finds a linear relationship between the percentage of cells in a monolayer corrected and the percent normalization of function (8).

Consequently, the simplest strategy to assure efficacy is to mimic the normal pattern of expression, *i.e.*, there should be a low level of expression per cell, and virtually every affected cell (100%) should be corrected. Is there an acceptable percentage below 100% of cells that might justify the initiation of a clinical trial? Given the likely difficulties in achieving gene transfer in man *in vivo* compared to any model system, certainly more than 10% of cells should be transduced in the most relevant model systems, *e.g.*, studies of human explants and pertinent animal models *in*

vivo. Unfortunately, none of the current *in vivo* model systems, such as the CF mouse, yield a sufficiently accurate lung infection phenotype to allow this critical question to be evaluated in a whole animal system.

Where are we in the clinic? Approximately 20 trials of CF gene therapy dosing the lung have been completed. These studies essentially have all been Phase I safety studies that have delivered both viral and nonviral vectors topically to the nose and/or lower airways via direct liquid instillation or via aerosol. With respect to adenoviral vectors, both single and multiple dosing studies have been performed.

From these Phase I trials, there has been a wealth of data produced on the safety aspects of first-generation nonviral and viral vectors. In brief, there have been no instances of identification and/or recovery of recombinant viruses from viral vectors, and relatively few if any DNA/vector-specific systemic effects resulting from intrapulmonary vector instillation have been detected. There have been reports of both inflammatory adverse events and immunologic responses to vectors. With respect to acute inflammatory responses, tachykinin-mediated neuroinflammatory responses in the nasal cavity in response to high-dose adenoviral vectors have been reported. A syndrome associated with acute pulmonary inflammation has also been reported (9). It is not clear what the etiology of this latter syndrome may be and whether it reflects, in part, deposition of vectors on alveolar versus airway surfaces, and/or the immune status of the patients. An acute, presumably cytokine-mediated response to liposome-mediated gene transfer in the lung has also been reported (E. Alton, personal communication). With respect to more delayed immunologic responses, rather small increases in adenoviral neutralizing antibody titers have been reported without an adverse clinical outcome (10). Although the data are more sparse, few or no inflammatory/immunologic responses have been reported with the AAV vectors.

With respect to gene-transfer efficiency/efficacy, perhaps the most quantitative data available are from studies that have dosed the nasal epithelium. For adenoviral vectors, initial reports from unblinded studies using nasal PD protocols that discriminated poorly between the CF versus normal phenotype indicated functional correction of CF epithelial Cl⁻ transport (11). Data from larger, placebo-controlled and blinded studies indicate

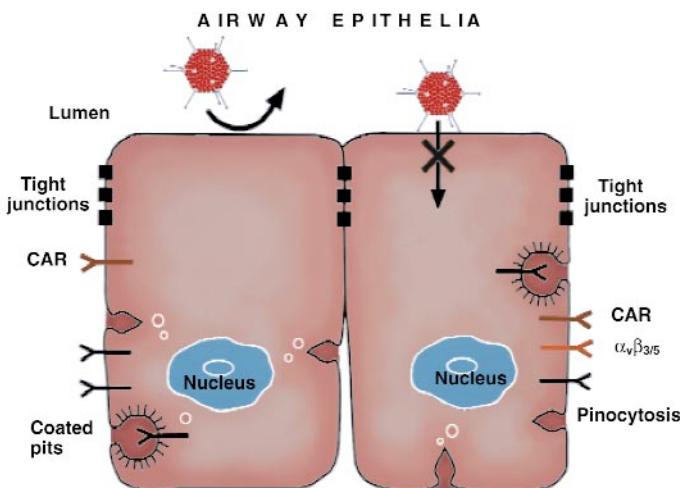


Figure 1

Barriers to vector-mediated gene transfer in WD columnar airway epithelial cells. The failure of vectors to bind to the apical membrane of WD cells is depicted on the left cell; the failure of "non-specifically" bound vectors to internalize is shown on the right cell. The tight junctions separate the apical cell membrane from the basolateral domain that selectively expresses specific viral receptors, *e.g.*, the CAR, "housekeeping" growth receptors, and integrins. WD, well-differentiated. CAR, Coxsackie virus and adenovirus receptor.

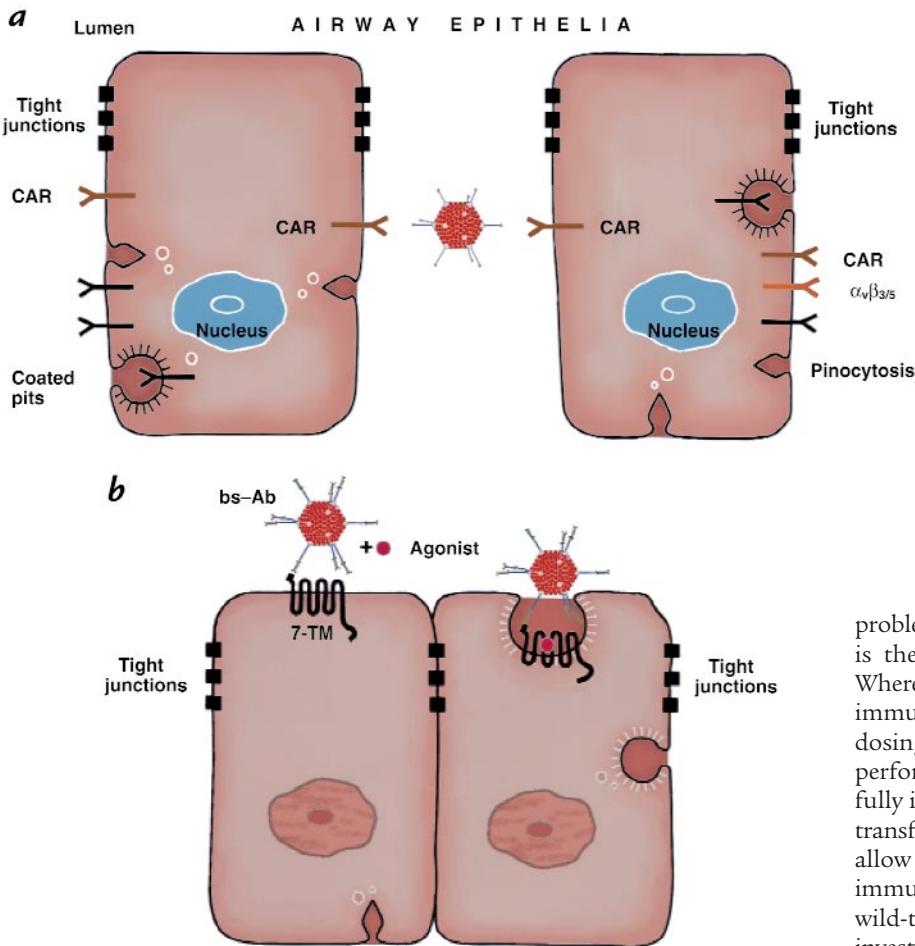


Figure 2

Strategies to increase adenovirus-mediated gene-transfer efficiency for well-differentiated columnar airway epithelial cells. (a) "Modification of the host." In this approach, the tight junctions are rendered permeable to vectors, which permits access to "vector-specific" receptors. (b) "Modification of the vector." A representative seven-transmembrane (7-TM) G-protein-receptor is shown that is activated to internalize via interaction with an agonist (denoted by purple-filled circle). Representative (adenovirus) vector is directed toward an external epitope of the receptor by bispecific antibodies (bs-Ab). Upon exposure to agonist, the 7-TM receptor is sequestered into a clathrin coated pit, carrying the vector into the cell.

that topical delivery of adenoviral vector to the nasal epithelium results in little gene transfer or functional correction, as measured with a combination of molecular (PCR) and functional (nasal PD) techniques (12). Similarly, there is little evidence of significant gene transfer with liposome-mediated gene delivery in the nasal cavity, using a variety of lipids and plasmid systems. The data with AAV in the nose are preliminary but also suggest poor efficacy.

Efficacy studies in the lower airways are more difficult to perform because of the difficulty in defining the precise sites of vector delivery and the inability to assess gene transfer quantitatively. With respect to adenoviral gene transfer, PCR assessment of gene transfer has detected wild-type CFTR transcripts in brushings from dosed CF airways but there are few quantitative data measuring the percent transduced epithelial cells in the region and no functional (PD) measurements of correction. With respect to liposomes, one nicely designed and performed study attempted to measure functional and molecular correlates of CFTR expression after aerosolized liposome-plasmid dosing of the lung. These investigators reported perplexing evidence for modest correction of Cl⁻ transport, but not Na⁺ transport function in the lung, without molecular (PCR) evidence of gene transfer (E. Alton, personal communication). Finally, although data from AAV administration to the lung are preliminary, they appear to show inefficiency as well.

What is the barrier to successful gene delivery? The major

problem confronting CF gene therapy is the inefficiency of gene transfer. Whereas studies of inflammation and immunologic consequences of vector dosing are important and should be performed, such studies will not be fully informative until adequate gene transfer efficiency is achieved. This will allow the complex inflammatory/immunologic picture of expression of wild-type CFTR in the CF lung to be investigated properly.

Inefficient gene transfer reflects the extremely effective adaptations of airway epithelia to prevent the penetration of foreign materials into airway epithelial cells or the interstitium. Airway epithelia create a complex series of barriers to prevent penetration of luminal materials, including both viral and non-viral vectors, into the cell or interstitial compartment. In series, these barriers comprise a well-defined mucus layer that may bind inhaled vectors and clear them via mucus clearance mechanisms, a glycocalyx that may bind vectors and prevent binding to cell surface receptors, and perhaps most importantly, an apical cell membrane that is relatively devoid of viral receptors and growth/tropic receptors that internalize as part of their biology (Fig. 1). This series of barriers is complemented by epithelial tight junctions that are "moderately leaky" to ions but quite "tight" for larger solutes, thereby preventing penetration by current vectors from luminal surfaces to the interstitium. Airway cells express most of the receptors that are used by current viral vectors for "virus-specific" entry on the basolateral membrane. Recent reports confirm that specific vector receptors, *e.g.*, the adenovirus receptor (13), the AAV receptor (heparan sulfate) (14), and the VSV receptor (15) are indeed localized to the basolateral membrane. In addition, most of the housekeeping/growth/trophic hormone receptors are also located basolaterally.

The early studies with model systems that employed poorly differentiated airway epithelial cells suggested that gene-transfer efficiency for a variety of vectors

would be high. However, with the advent of the use of well-differentiated (WD) culture systems, supplemented by freshly excised organ culture systems, it became clear that a common theme was emerging: *a*) that virtually all vectors (viral and nonviral) did not bind to the apical (lumenal) surfaces of WD airway epithelial cells; and *b*) that apical surfaces of WD airway epithelial cells have a low basal and stimulated rate of endocytosis (13, 16).

What is the answer to increase efficiency? It is apparent that novel strategies must be adopted to increase gene-transfer delivery. As mentioned above, strategies that may use the vascular compartment as a dosing route should be explored but the difficulties in overcoming the large number of barriers between the vascular compartment and airway epithelial cells — endothelial cell, endothelial cell basement membrane, interstitium, and epithelial basement membrane — make this route challenging. With respect to intraluminal dosing of the superficial epithelium, at least two general strategies can be envisioned to increase gene-transfer efficiency.

In a strategy termed "modification of the host," it may be rational to reduce the barrier functions of epithelial tight junctions so that vectors can penetrate to the basolateral membrane of target cells that, as indicated above, are naturally rich in viral and other internalizing receptors (Fig. 2). Abrogation of tight junction barrier function can be achieved by non-specific damage, such as has been demonstrated with oxidant gases (17) and surface-active adjuvants, *e.g.*, detergents (18). Such strategies have been shown to increase gene-transfer efficiency in airways of rodents dramatically, but it likely will be difficult to titrate down the dose of an oxidant gas and/or deliver a specific mass of a detergent safely to make this strategy therapeutic for CF patients. More specific modifications of tight junctional permeability through cellular regulatory mechanisms thus are more appealing. Increasing knowledge of the cellular regulation of tight junctional permeabilities, including the interrelationships between the adherens junction and the tight junction, may make this approach feasible. The ultimate goal is a safe and effective strategy, which depends on: *a*) transient, reversible permeabilization of tight junctions; and *b*) permeabilization of tight junctions without producing inflammation, hence avoiding vascular leak into the airway lumen and airways irritation.

The alternative approach is to "modify the vector." The concept here is to direct a vector to a "target" expressed on the apical cell membrane that has the capacity to both bind and internalize a vector. Identification of suitable targets in the airway has not been easy because of the paucity of expressed receptor/membrane proteins on airway epithelial surfaces that internalize as a function of their biology. However, there is a class of receptors that normally mediates acute airway epithelial cell responses to the luminal environment, *i.e.*, seven transmembrane receptors. Several members of this class are expressed on the lumen of human airway cells.

Perhaps the most attractive target from the point of view of the level and extent of expression in the airways is the extracellular ATP/UTP receptor, termed P2Y2-R. This receptor internalizes into the cell via clathrin coated pits upon agonist stimulation. Many viruses have evolved mechanisms and escape from clathrin coated vesicles via a

process termed endosomolysis. Preliminary studies in non-polarized cells have documented that either bi-specific monoclonal antibodies directed towards engineered epitopes into the external domain of P2Y2-R or modifications of the native ligand (BiotinUTP) to direct vectors to P2Y2-R can produce efficient gene transfer via this pathway (19). More importantly, vector internalization and gene transfer can also be achieved when P2Y2-R is expressed in the apical membrane of WD cells. Targeting through this approach offers several attractive features, including a wide versatility with respect to the targeting molecules themselves, *e.g.*, antibodies, peptides, or modified ligands, and the ability to link the targeting molecule to a wide variety of vectors, including plasmids, adenoviral, AAV, and lentiviral vectors.

Can we select a preferred vector now? This would be premature. A nonviral vector might be preferable because of the simplicity of the system, but a viral vector, if it were sufficiently safe and efficient, could be a viable alternative. It is likely that we will see a series of both types of vectors developed and used clinically. For example, it is possible that host modification or vector luminal targeting will become a reality relatively rapidly, and that "high-capacity" adenoviral vectors, because of their proven ability to express in airway epithelial cells, their relative safety, and the transient nature of their expression, would be optimal for new studies of safety and efficacy. In the long term, it would appear reasonable that for a genetic disease like CF, integrative gene transfer will be preferable. Thus, it appears wise to continue the development of lentiviral vectors, both HIV (20) and non-human (21), and AAV vectors for this use.

The future. It is clear from analysis of the data describing gene-transfer efficiency from the reported clinical studies that an order or two of magnitude increase in efficiency will be required for gene transfer to be therapeutically relevant in CF. The good news is that all of the previous work has in principle identified the hurdles that must be cleared prior to initiation of novel strategies in man. For example, the WD cultures, freshly excised explant cultures, and biologic and expression studies in the mouse nose (but not infection phenotype in the lung) appear to be accurate models for predicting efficacy in man *in vivo*. Further, although there have been questions about its relevance, it does appear that the nose as a first approximation is a good model for lower airways gene transfer in man. Thus, the trial designs in the nose that have been generally agreed upon, *i.e.*, double-blind placebo-controlled studies using nasal PD protocols designed to measure basal Na^+ transport and Cl^- transport, coupled to molecular and morphologic studies with a spectrum of sensitivities, appear to offer a rigorous way to assess the efficacy of a new strategy before initiating more difficult studies in the lung.

A challenge for lung gene transfer, like other forms of CF lung therapy, will be the initial trial design to measure efficacy. Here again, much progress has been made. In the context of exploring drug therapy to treat the initiating cause of disease, trial designs have been explored to assess the ability of novel therapies to protect the lung against disease. Important analyses of the required sample sizes for these studies as a function of patient age have also been recently reported, and healthy discussions on surrogate markers in the lung

are ongoing (22). Thus, one can be optimistic that when we develop strategies that promote routinely between 10% and 100% gene-transfer efficiency in human airways, we will be smart enough not to miss the clinical benefits of gene transfer in CF patients.

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