

TOWARD CYSTIC FIBROSIS GENE THERAPY

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KEY WORDS: adenovirus, adeno-associated virus, cystic fibrosis transmembrane conductance
regulator, liposome, vector

ABSTRACT

Cystic fibrosis (CF) is a common genetic disorder characterized by defective epithelial chloride transport and progressive lung disease. Although great strides have been made in the treatment of CF, it remains lethal, often by early adulthood. CF is one of the most extensively researched genetic diseases as a target for gene therapy development. It may also serve as an important model for gene therapy of other diseases. Preclinical and clinical research has led to the rapid development of a variety of vectors designed to correct the basic defect in CF, including adenovirus, adeno-associated virus, and liposomes. Clinical studies have identified the host immune response and low vector efficiency as key impediments to effective CF gene therapy. Further research promises to refine vector technology and bring CF gene therapy to the bedside.

INTRODUCTION

Cystic fibrosis (CF) is a common, lethal, genetic disease and is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). The main characteristics of CF include chronic bacterial infections of the upper and lower respiratory tract with resulting lung damage, exocrine pancreatic insufficiency, and abnormal regulation of salt transport across the gastrointestinal and respiratory epithelium. Inflammation of the respiratory tract and chronic bacterial colonization, particularly with *Pseudomonas aeruginosa*, mark the early course of CF lung disease (1). Over time, airway injury second-

dary to inflammation results in bronchiectasis and subsequent progressive respiratory failure.

The disease is transmitted through autosomal recessive inheritance and afflicts 30,000 patients in the United States (2). CF heterozygotes, present in the Caucasian population at a frequency of 1 in 25, have no known clinical syndromes nor detectable laboratory abnormalities. The reason for the high heterozygote frequency in the general population is unknown, but it may be related to protection from infant diarrheal disease (3).

CF is a multisystem disease involving exocrine dysfunction in all affected organ systems. CF involves the respiratory system, the gastrointestinal system, the endocrinologic system, the musculoskeletal system and skin, and the genitourinary system. The most severe manifestations of CF are in the respiratory system, especially the lower respiratory system. Prior to 1960, the median length of survival of CF patients was less than five years. Since then, dramatic gains have been observed, likely related to increasingly effective treatment of bronchopulmonary infections and more effective airway clearance techniques. Since 1990, the median years of survival has been in the high 20s. In 1994, it was 28.3 years (4). Despite important therapeutic advances, definitive treatment remains elusive. CF is an attractive disease for gene therapy because it does not have adequate treatment options, and because it is relatively common, lethal, and monogenic (5). The gene therapy strategy for CF involves complementation or augmentation (6) of the mutant alleles with wild-type CFTR. This review focuses on current gene therapy technology and early clinical results. More detailed reviews are available (5, 7–19).

CF BIOLOGY

How do mutations in CFTR lead to the clinical manifestations of the disease? Despite great strides in understanding the molecular biology and physiology of CFTR, the detailed pathophysiology of CF remains incompletely understood. However, a picture of CF pathophysiology is emerging and will be instrumental in the design and optimization of CF gene therapy.

Although the autosomal recessive inheritance of CF has been known since the 1940s, the genetic defect was not identified until 1989. CF mutations were localized to a 250,000-bp gene on chromosome 7 by positional cloning (20). The gene product is a 1480-amino acid, transmembrane protein called the CFTR (cystic fibrosis transmembrane conductance regulator) because of its role in chloride secretion (21). The most common mutation in CF, a 3-bp deletion in exon 10 resulting in the deletion of a phenylalanine at position 508 in CFTR protein ($\Delta F508$), accounts for 70% of CF alleles (22). CFTR is a chloride channel regulated by cAMP-dependent protein kinase and adenosine

triphosphate (23). All of the defined mutations in CFTR result in aberrant chloride secretion through defective protein production, defective protein processing, defective regulation, or defective chloride conduction (23). CFTR also interacts with epithelial apical sodium channels, resulting in increased sodium conductance in airway epithelial cells with mutant CFTR (24). The combination of defective chloride secretion and increased sodium absorption may lead to dehydration of epithelial surface liquids and leads to the inspissated secretions and impaired mucociliary clearance in the lung that characterize the pathophysiologic complications of CF (1). CFTR may have other cellular roles, including regulation of intracellular organelle acidification (25), sialylation of cell surface receptors (26), and sulfation of high-molecular-weight glycoconjugates (27). How these other roles for CFTR contribute to the overall pathophysiology of CF remains incompletely understood.

Clinically, the site of the most severe pathophysiology is in the respiratory system (1). In fact, over 95% of deaths from CF can be attributed to lung disease (4). Pulmonary pathophysiology starts in the peripheral airways. CFTR dysfunction leads to altered airway electrolyte regulation and fluid secretion dysfunction. CF airway secretions are thick and have increased viscosity. In normal airways, a concerted process of secretion and absorption of fluid helps rid the lungs of particulates, including inorganic debris, airborne bacteria, and viruses, via mucociliary clearance. In CF airways, this process is dysfunctional. Particulates are not efficiently cleared from airways; inflammatory reactions eventually result in mucus plugging, which in turn results in obstruction. Hyperinflation and airway obstruction contribute to bronchiectasis. In addition, CF patients become selectively infected with a few types of bacteria, including *Staphylococcus aureus*, *Hemophilus influenzae*, and *P. aeruginosa*. Chronic bronchopulmonary infection ensues, which is the prominent cause of lung damage, pulmonary dysfunction, and death. The molecular physiology behind increased infection in CF remains unclear, but it may be related to altered epithelial cell uptake or adherence of bacteria (28) and decreased effectiveness of local endogenous antimicrobials (29).

GENE THERAPY BIOLOGY

The feasibility of gene therapy for CF was demonstrated soon after the defect was cloned (21). Chloride transport was restored to tissue-cultured CF epithelial cells after transfection with wild-type CFTR (30, 31). Gene transfer with wild-type CFTR also corrects chloride transport in transgenic animal models of CF (32, 33). Since chloride transport is thought to be critically involved in the pathophysiology of CF, these observations set the stage for future gene therapy endeavors.

Because the most severe pathophysiology of CF is in lung, most CF gene therapy efforts concentrate in the respiratory system. Direct lung gene therapy prevents the use of an *ex vivo* strategy—where cells are harvested, genetically modified, and returned to the body—as is used for adenosine deaminase deficiency (34). Instead, an *in vivo* approach must be used; gene therapy vectors must be directly applied to lung epithelial tissue resulting in expression of wild-type CFTR. Clinical trials of gene transfer agents often use the upper respiratory system, including nasal and maxillary sinus epithelium, for more convenient testing of gene therapy, but ultimately, aerosol delivery to the whole lung is the goal of most current CF gene therapy efforts (11, 16).

Which lung cell type or types must be targeted to effect clinical benefit in CF? The appropriate cell type for CFTR gene transfer remains an open question. Wild-type CFTR expression is highest in submucosal gland epithelial cells residing in the proximal airway, but surface epithelial cell expression is also observed at a lower level (35, 36). In addition, some surface epithelial cells in the distal airway express CFTR as well as alveolar cells at even lower levels (37). Ideally, long-term CFTR gene transfer to airway progenitor cells would be the most effective, but the identity and biology of progenitor cells remain controversial (38, 39). Because of technical limitations restricting aerosol delivery of gene transfer vectors to airway surface epithelial cells, most gene therapy strategies are currently aimed at gene transfer to them not to submucosal or progenitor cells.

The *in vivo* gene therapy approach required for CF makes special demands of a gene delivery system. The ideal CF vector would be completely safe, highly efficient for entering cells and expressing CFTR, tropic for airway epithelial cells, capable of transducing nondividing cells as are found in the surface airway, and of low immunogenicity. The ideal vector has not yet been realized, but two types of vectors—viral and nonviral—have been extensively researched and each provides a unique set of advantages and disadvantages. Viral vectors are vehicles for expression based on existing viruses, but they are genetically modified to be replication deficient and they contain an expression cassette with CFTR cDNA and an appropriate promoter. Typically, viral vectors have higher efficiency but are more immunogenic than nonviral vectors. Viral vectors developed specifically for use in CF include adenovirus and adeno-associated virus, although other viruses have been considered. Nonviral vectors are generally a combination of CFTR cDNA with a suitable promoter and lipid, protein, or other molecules, which aids in the uptake of nonviral vectors by target cells. In contrast to viral vectors, nonviral vectors are typically less efficient and less immunogenic. Nonviral vectors developed specifically for CF include the combination of lipid and DNA called liposomes. A comparison of specific vectors is shown in Table 1 and is discussed below.

Table 1 Comparison of gene therapy vectors for cystic fibrosis^a

Determinant	Viral			Nonviral	
	Adenovirus	AAV	Retrovirus	Liposome	Molecular conjugate
Composition	dsDNA	ssDNA	ssRNA	DNA plus lipid	DNA plus polypeptide
Genome (kb)	36	5	10–20	NA	NA
Carrying capacity (kb)	7–8	<5	6–7	>10	>10
Transduction	Dividing and nondividing	Dividing and nondividing	Dividing only	Dividing and nondividing	Dividing and nondividing
Efficiency	High	Intermediate	Intermediate	Low	Low
Expression	Transient	Intermediate	Long-term	Transient	Transient
Integration	No	No ^b	Yes	No	No
Tropism	Yes	Yes	No	No	Yes
Immune response	High	Low	Low	Low	Low

^ads, Double stranded; ss, single stranded; NA, not applicable.

^bAAV vectors persist largely as episomal DNA, but a small amount of AAV vector may also integrate in some conditions.

Because current vector technology is inefficient, a key question is how much CFTR must be transduced in order to have a beneficial effect on the disease. CF is a recessive disorder, and heterozygotes with one mutant CF allele do not have clinical disease (1). Other clinical data suggest that some phenotypically normal individuals have up to 92% abnormally spliced CFTR mRNA, which produces a defective protein product (40, 41). Similarly, pre-clinical experiments in monolayers of CF epithelial cells suggest that a minimum of only 6% of cells require wild-type CFTR expression in order to correct the chloride transport defect (42). On the other hand, close to 100% of cells require wild-type CFTR expression in order to normalize increased sodium conductance (43). If correction of chloride secretion is all that is necessary for a beneficial effect, then as little as 6–8% of airway cells will require wild-type CFTR expression; however, if other CFTR functions, like regulation of epithelial sodium channels, are necessary for clinical benefit, then a much higher percentage of cells will require transduction. Transgenic CF animal models may provide a more detailed estimate for the minimal amount of CFTR expression resulting in clinical benefit. Unfortunately, most transgenic CF models do not exactly mimic the respiratory disease process, making interpretation of these studies challenging (33). Another key question related to the amount of CFTR expression is whether too much expression is deleterious for cells. Excessive CFTR expression is toxic in selected *in vitro* experiments (44), but these observations have not been confirmed with *in vivo* animal models (45).

Delivery and evaluation of the effects of gene transfer agents is another dilemma. A comparison of current gene therapy delivery techniques is displayed in Table 2. Completed studies of CF gene therapy have been confined to nasal mucosa (46–48) or lung subsegments (49). Key advantages of testing gene therapy on the nasal mucosa are accessibility, local application, safety to patients, and the ability to measure a physiologic correlate of CFTR function, the nasal potential difference (50). However, the nasal mucosa is not significantly affected clinically in CF patients; therefore, assessment of clinical outcomes of gene therapy to the nasal mucosa is not possible. Delivery of vectors to a lung subsegment can assess safety of gene therapy in the tissue, which must ultimately be therapeutically targeted, but measurement of clinical efficacy of gene therapy in lung subsegment is difficult. In addition, measurement of biologic efficacy is invasive secondary to the requirement for bronchoscopic retrieval of cells for examination (49). Studies of clinical efficacy in the whole lung will require aerosol delivery of large amounts of vector and large numbers of patients. Alternatively, the maxillary sinuses may prove to be a more useful testing ground for gene therapy, especially with regard to clinical efficacy, because sinuses of selected patients develop disease similar to that of the lung (51).

GENE THERAPY VECTORS FOR CF

Gene transfer vectors for CF have not followed the traditional pattern of drug development observed for other pharmaceuticals. One key difference between drug and gene transfer vector development is that drugs are often a single chemical entity extensively screened preclinically, while gene transfer vectors are numerous and rapidly evolving in effectiveness and other features. Thus,

Table 2 Comparison of delivery techniques for CF gene therapy^a

Feature	Nose	Maxillary sinus	Lung subsegment	Whole lung
Accessibility	+++	++ ^b	+	+
Safety to patient	+++	++	+	+
Local application	Yes	Yes	No	No
Clinical outcomes	No	Yes	No	Yes
Safety outcomes	Yes	Yes	Yes	Yes
Potential difference measurement	Yes	Yes ^c	No	No
Vector requirement	+	+	++	+++

^a+++ , Greatest; ++, intermediate; +, least

^bThe maxillary sinuses are easily accessible only in patients who have undergone bilateral maxillary anastomy for treatment of chronic sinusitis.

^cPotential difference measurements are confounded by reductions in potential difference by increases in inflammation from, e.g. infection.

Gene therapy development and clinical trials are occurring on multiple parallel tracks. Several viral and nonviral vectors have reached clinical trials, and information gleaned at the bedside has only increased the pace of further development of new and modified vectors. This review concentrates on those vectors that have already reached clinical trials (46–49, 52–61).

Viral Vectors

Adenovirus was an early favorite candidate vector for CF and is a double-stranded DNA virus, which naturally infects the respiratory and gastrointestinal tracts of humans. A number of advantages recommended it as a gene transfer agent, especially tropism for airway epithelium, ability to produce high viral titers, and ability to transduce nondividing cells. Feared disadvantages included transient expression of the transgene, recombination of the modified virus with wild-type viruses, shedding of the modified virus, and host immune response to the transgene or modified virus.

Adenovirus serotypes 2 and 5 are tropic for respiratory epithelium, and hence, vectors are based on these serotypes. An early hurdle was engineering replication-deficient recombinant virus, necessary for safe administration to humans (19, 62–64). This was accomplished by deleting early genes that code for regulatory proteins, leaving intact late genes that code for virus structural proteins. Since the early regulatory genes control activation of the late genes, deletion of early genes allows late genes to remain dormant and, thus, prevent replication.

First-generation, replication-deficient adenovirus vectors contain an immediate early gene (E1) deletion plus the cDNA of CFTR or a marker transgene (19, 62–64). First-generation adenovirus vectors are effective for expression of CFTR and other marker genes in both in vitro and in vivo animals studies (62, 63, 65–68). Safety studies varied from no toxicity in rodents and rhesus monkeys (68) to lung inflammation in baboons (69).

Human clinical trials were necessary to further evaluate biologic efficacy and safety. All published human trials using nasal delivery of first-generation adenovirus vectors showed variable, transient expression of the CFTR transgene (Table 3). Two of three published single nasal administration human trials with first-generation adenovirus vectors showed variable, transient functional restoration of chloride transport as measured by nasal potential differences (48, 70). The third study was a rigorous, double-blind, placebo-controlled investigation of a first-generation adenovirus vector that failed to show any functional restoration of chloride transport despite use of very high virus concentrations (47). Concerns regarding recombination of the modified virus with wild-type strains and shedding of the modified virus were not realized (47–49, 54). However, transient expression and the host immune response plagued most clinical trials using first-generation adenovirus vectors (47, 49,

54, 70). Host immune response was especially prominent in one lung subsegment trial at the highest dose of adenovirus vector used (49). The transient expression provided by adenovirus vectors would require repeated administrations; however, repeat administration of a first-generation adenovirus vector produced increased immune responses and decreased correction of chloride transport (54).

The combination of immune response and transient expression have dimmed hopes that first-generation adenovirus vectors will be used for treatment of CF. Modifications of first-generation adenovirus vectors, second- and third-generation adenovirus vectors, contain further deletions of early genes in an attempt to further reduce or eliminate expression of late viral genes and, thus, reduce the host immune response (19, 71–74). In vitro and animal studies of next-generation adenovirus vectors are encouraging, but further work will be necessary to investigate host immune responses to these agents. A second strategy is to provide transient immune suppression around the time of vector

Table 3 Viral vector gene therapy clinical protocols^a

Institution	Vector	Delivery	Dosing	First patient	Expression	Function	Ref.
Yale/Cornell	Ad type 5 E1–, E3+	Nose, subsegment	Single	4/17/93	Yes	Yes	49, 70
Iowa/ Genzyme	Ad type 2 E1–, E3+	Nose, subsegment	Single	7/26/93	Yes	Yes	48
Michigan/ Pennsylvania	Ad type 5 E1–, E3+	Subsegment	Single	9/3/93	—	—	52
North Carolina	Ad type 5 E1–, E3+	Nose	Single	1/11/94	Yes	No	47
Cincinnati/ Genetic therapy ^b	Ad type 5 E1–, E3+	Nose, subsegment	Single	9/21/94	—	—	53
Iowa/ Genzyme ^c	Ad type 2 E1–, E3+, E4–	Nose and maxillary	Repeat	4/20/94	Yes	Yes	54
Cornell	Ad type 5 Ad E1–, E3–	Subsegment	Repeat	2/5/95	—	—	55, 56
Massachusetts/ Harvard/ Genzyme	Ad type 2 E1–, E3+, E4–	Subsegment Whole lung	Single	2/21/95	—	—	57
Genzyme	Ad type 5 Ad E1–, E3–	Nose, whole lung	Single	NA	—	—	58
Johns Hopkins/ Targeted Genetics	AAV	Nose, subsegment	Single	11/21/95	—	—	59
Harvard/ Targeted Genetics	AAV	Maxillary sinus	Single, repeat	12/16/95	—	—	60

^aU, University; Ad, adenovirus; E, early gene; NA, not available.

^bRevised to nose only.

^cOnly nose reported.

administration (19, 75–77). Although this strategy appears successful in animal models, the clinical utility of even transient immune suppression in CF patients plagued by chronic bacterial infection remains uncertain (16).

Another strategy is to turn to a different viral vector system. Adeno-associated virus (AAV) is a naturally replication-deficient single-stranded DNA parvovirus that depends on coinfection with a helper virus, such as adenovirus, for replication and is not associated with any known human disease (8). AAV vectors may offer theoretical advantages over adenovirus vectors, including lack of the pathogenicity of wild-type virus as well as modified AAV and longer-lasting expression. In vitro and animal studies have shown that CFTR transcripts and the CFTR protein can be detected up to six months after transduction with an AAV vector (78). Like adenovirus vectors, AAV vectors are tropic for airway epithelium, able to produce sufficient viral titers (though less than adenovirus produces), and able to transduce nondividing cells (79). These features suggest that AAV vectors may perform well as a CFTR gene transfer agent.

AAV is naturally replication deficient, but an additional technical challenge specific to AAV vectors is the small size of the viral genome and consequent limitation of transgene insert size (80). To make a CFTR AAV vector, the viral genes necessary for replication and encapsidation, *rep* and *cap*, respectively, must be deleted in order to fit the CFTR transgene. Because CFTR cDNA is at the upper limit of insert size, promoter selection was problematic. Fortunately, the left-hand terminal repeat, already necessary for packaging and excision of the recombinant vector, has sufficient promoter activity for transgene expression. For production, *rep* and *cap*, along with a helper virus such as adenovirus, must be provided to make recombinant virions. Besides the limitation in insert size, other AAV disadvantages include possible vector integration, rescue or recovery of virus after infection, and the recently observed requirement for helper virus infection for leading strand synthesis, which in turn limits transgene expression (81, 82). Although, in the absence of helper virus, wild-type AAV integrates into a site on human chromosome 19 (8, 83), AAV vectors predominantly persist in episomal form in vitro and in vivo (83, 84). Despite the relative lack of integration in host chromosomes, an AAV vector can still be rescued from rhesus monkey airway epithelium by coinfection with wild-type AAV and adenovirus (84). The rescue of an AAV vector may portend a risk of disadvantageous shedding after gene therapy treatment. Despite the evidence for shedding, animal experiments have not demonstrated pathologic or immune alterations after AAV vector transduction. Viral shedding, safety, biological efficacy, and other issues are currently being further examined in two ongoing human phase I gene therapy trials (Table 3).

Because neither adenovirus nor AAV vectors fulfill all the criteria for the ideal vector, other viruses are also being considered as potential vectors. Retrovirus vectors, already used in other types of gene therapy studies, were initially spurned as a CF gene therapy vector because they require dividing cells for efficient transcription. However, recent studies have shown that the rate of proliferation in CF airway is much higher than normal, lending support to the notion that retroviruses may yet have a role in CF gene therapy.

Nonviral Vectors

Viral systems are not the only potential method for CFTR gene transfer. Two main kinds of nonviral vectors are in development: liposome-DNA complexes and molecular conjugates. The best-studied liposomal gene transfer method is based on cationic lipid vesicles that bind negatively charged DNA and fuse to cell membranes for DNA transfer (85). The DNA consists of CFTR cDNA plus a promoter together on a plasmid (86). The main advantages of liposomal gene transfer over viral gene transfer are the greater safety of liposomes and ease of preparation. Liposomal treatment has been shown to be safe in a variety of animals and humans (87). The main disadvantages are decreased transduction efficiency compared with viral vectors, lack of tropism for airway epithelium, and the transient nature of expression driven by liposomal vectors.

Liposomal-mediated gene transfer has been effected in a variety of *in vitro* systems and has also been shown to correct the ion transport defect in CF mutant mice (32, 86). A phase I, randomized, double-blind clinical trial investigating liposomal transfer of CFTR to the nasal mucosa in CF patients assured the safety of this gene transfer technique (46). Variable and transient expression as well as functional restoration were observed in this trial. Three human clinical trials using liposomal gene transfer are ongoing (16). Observed expression and functional restoration by the liposomal vector are likely not adequate for CF gene therapy. Disappointing efficiency is driving the search for more effective combinations of lipids and plasmids (17).

One disadvantage of liposomal-mediated gene transfer is the lack of tropism for airway epithelium. Molecular conjugates are designed to take advantage of receptor-mediated endocytosis and, thus, to provide some specific delivery capabilities to this nonviral gene transfer technique. One conjugate approach cleverly uses adenovirus virions complexed with polylysine-condensed DNA to augment the uptake and expression of DNA (88, 89). This complex of adenovirus particles, polylysine, and a DNA plasmid is taken up by transferrin receptors. Encouraging results were obtained *in vitro*, but they have not been confirmed in animal models (90). Despite inefficient expression *in vivo*, this delivery system has the advantage of targeting specific cell types by exploiting cell-specific receptors. No clinical trials using molecular conju-

gates are ongoing. A variety of other molecular conjugates are being developed that use engineered viral peptides (91) and specific antibody fragments (92), among other strategies.

CONCLUSION

Recent years have seen an explosion of research toward CF gene therapy. A fertile combination of preclinical and clinical research is producing rapid progress in defining the molecular pathophysiology of CF and improving vector technology for producing CFTR expression. Gene therapy clinical trials have refined our understanding of the barriers to CFTR gene transfer, and they will continue to impact the development of future generations of vectors in an iterative process involving both preclinical and clinical research. High expectations of gene therapy by investigators, clinicians, patients, and the lay public should not divert attention from the rapid progress that has already been made, nor should it dampen enthusiasm for the necessarily iterative process of gene therapy development.

ACKNOWLEDGMENTS

This work was supported by NIH grant M01 RR00070 and by a grant from Cystic Fibrosis Research, Inc. We thank all our colleagues who shared preprints. Our apologies to any investigator whose work was not cited because of the required brevity of this review.

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