UNDER THE MICROSCOPE

Cystic Fibrosis: More Questions than Answers

Janet E. Larson, MD

Head, Ochsner for Childrens Research Institute, Department of Pediatrics, Ochsner Clinic Foundation, New Orleans, LA

An examination of the changing historical presentation of cystic fibrosis (CF) offers insight into the problems of the research and clinical treatment of a complex disease. When the sequence of the cystic fibrosis transmembrane regulator (CFTR), the gene responsible for CF, was determined in 1989, many optimistically predicted a cure for the disease with gene therapy, because the lung, the primary organ affected by CF, is easily accessible. Over the past decade, clinical trials have been unsuccessful, and while many blamed the failure on emerging vector technology (the vehicles used to transport the gene of interest into the target cell), a poor understanding of CF biology was also a major contributing factor. CFTR's exact role in the airway and the mechanism for its direct participation in the disease pathology still remains unclear. This is not surprising when one examines the changing face of this complex disease.

CF is a pleiotropic disease with a highly variable clinical presentation. Not only do affected individuals show great differences in their disease presentation, but the clinical definition of CF has changed over time. Cystic fibrosis was originally described in 1938 as "mucoviscidosis," a disease of the pancreas (1). Before and during the 1950s, infants born with meconium ileus (the intestinal blockage pathognomonic for CF) or that failed to thrive during infancy had a 45% mortality rate by 1 year of age. At that time CF was perceived as a pancreatic and gastrointestinal disease because affected infants did not survive long enough to develop overt lung disease. When survival improved due to early recognition and pancreatic enzyme replacement therapy, the incidence of fatal pulmonary complications increased and CF was recognized as a lung disease. With prolonged survival (although the mean survival is still only 32 years), CF patients require liver transplantation for cirrhosis and portal hypertension, and their health is further complicated by osteoporosis.

THE PARADOXES OF CYSTIC FIBROSIS

The complex clinical presentation of CF is caused by the lack of a protein whose function is equally complex. In the 1950s it was observed that affected individuals had excessive salt loss. This led to the development of the chloride sweat test as a primary diagnostic tool in 1959. However, the direct correlation of disease pathology to the absence of a chloride channel still remains elusive. Despite the discoveries of CFTR's many cell regulatory functions (which include the release of extracellular ATP), the pathologic symptoms of CF continue to be defined in terms of a loss of a chloride channel. Increased bacterial adherence and colonization in the lung are thought to be the direct effect of abnormal sodium, potassium, and chloride flux into the airway epithelium. Thickened mucus, thought to be present because of decreased airway water, promotes defective ciliary clearance. The observations contradicting this hypothesis are frequently dismissed as changes resulting from chronic infection.

However, chronic infection cannot explain all of the paradoxes related to this protein and its relationship to the disease, some of which are shown in Table 1. CFTR has been shown to regulate many other proteins and ion channels. Often CFTR's direct actions in an in vitro (tissue culture) system are different than those observed in tissue from CF patients. The epithelial sodium channel (EnaC) is down-regulated by the presence of CFTR in tissue culture.

Enhanced sodium absorption is present in the airways and colon of CF patients but is absent in the sweat glands despite the expression of ENaC in all of these tissues. The calcium-regulated chloride channel is also down-regulated by the presence of CFTR in tissue culture. In CF patients, the channel activity remains intact in the airways but is defective in the intestine. These ion channels show a range of activity dependent on the organ examined, despite the absence of functional CFTR activity in these tissues.

After the gene was cloned and the expression of CFTR mRNA was localized, more paradoxes were discovered. The expression of CFTR mRNA was not present in "affected" cells but was present in "non-affected" cells. Myocardial and renal functions appear normal in CF patients while both of these organs express CFTR. In contrast, the goblet cell (a major airway secretory cell) secretes defective mucus, yet has undetectable levels of CFTR. Although there is moderate expression in the adult intestine and pulmonary submucosal glands, on average only two copies of CFTR mRNA are present in each lung epithelial cell.

To date, there is no general concept of how or why CFTR controls cellular functions such as mediation of vesicular trafficking, modulation of intracellular pH, and regulation of glycosylation (2). To complicate matters, there are subsets of patients with the CF phenotype and no mutation in the CFTR gene. Recently, Groman and coworkers described 74 patients with CF pathology that had only one or no mutations in CFTR. Half of these patients had normal chloride sweat tests, yet had CF pathology in one or more organs (1).

NEW PERSPECTIVES

Is there any unifying hypothesis that can explain all of these paradoxes? When our laboratory began studying CF in 1995, we were unaware of the complex history associated with the disease. We had devised an in utero gene transfer technology to circumvent the problems of inflammation seen with adenoviral-mediated gene therapy (3). During the course of these experiments, it was discovered that the in utero transfer of the CFTR gene to normal rat fetuses resulted in phenotypic changes in the lungs of neonatal pups. At the time of gene transfer, the targeted epithelial cells were undifferentiated somatic stem cells. Administration of the CFTR gene to this epithelium using an adenovirus vector system resulted in persistent phenotypic changes in cells, although the expression of the transgene was transient. These data provided the first insight that CFTR expression during the fetal period could permanently alter the differentiation of lung epithelial stem cells. The permanent functional changes in the in utero CFTR-treated rats included an enhanced resistance to pulmonary bacterial infection 3 months after birth (4).

At the same time, other laboratories were examining the temporal and tissue-specific expression of CFTR (5). CFTR lung expression is greatest during the fetal period when it is localized to airway epithelial stem cells. As these multipotential stem cells differentiate, the expression of CFTR dissipates and the adult lung expresses only a fraction of that expressed during the fetal period. Thus, CFTR resembles other developmentally important genes in its expression at specific times during organogenesis.

In addition to its recognized role as a chloride channel in the mature lung, CFTR's expression in undifferentiated epithelial cells suggested another role(s) during development. Moreover, this raised the question of how much of CF disease pathology could be attributed specifically to the lack of CFTR expression during differentiation and how much could be attributed to lack of a chloride channel in the mature lung.

These questions prompted further experiments by our laboratory in the CF knockout mouse. Reversal of the lethal phenotype meconium ileus in the CF (cftr-/-) mouse following transient in utero expression of CFTR confirmed the role of this gene in gut development. Because of the rapid cell turnover, the human CFTR transgene was detected in the fetal gut for up to 72 hours post-treatment but not after birth. The in utero gene therapy did not permanently replace the CFTR-encoded cAMP-dependent chloride channel but rescued the mice from the disease phenotype and reversed biochemical markers specific to the knockout phenotype. These data established that extrauterine expression of CFTR was not required for the correction of the intestinal obstruction in *cftr* -/- mice (6).

Many other regulatory proteins have multifunctional roles during or after development and cellular differentiation. The biological effects of these proteins occur only transiently but remain a necessary link in a developmental cascade requiring a series of carefully

Table 1. Paradoxes between the functions of cystic fibrosis transmembrane regulator and the pathophysiology of cystic fibrosis (2).

Cellular Function Affected by CFTR	Direct Protein Interactions in vitro	In vivo Observations
Epithelial sodium channel	Down-regulated by CFTR	Enhanced sodium adsorption in the airways and colon of CF patients but absent in sweat glands
Calcium-regulated chloride channel	Down-regulated by CFTR	Activity intact in CF airways chloride channel but defective in CF intestine
Outwardly rectifying chloride channel	Stimulated by CFTR	Activated in wild-type cells but not in CF cells. No correlation between CFTR mRNA levels with ORCC activity in numerous cell types
Glycoprotein processing	Increased sulfation and fucosylation with decreased sialylation	Altered glycosylation of secretory products of goblet cells. Undetectable levels of CFTR gene expression in goblet cells

**CFTR = cystic fibrosis transmembrane regulator; CF = cystic fibrosis; ORCC = outwardly rectifying chloride channels*

orchestrated events. For example, transforming growth factor-beta-1 (TGFB), a protein recognized as a key participant in growth regulation and cellular differentiation, is compared to CFTR in Table 2. TGFB's pleiotropic effects result from its role in signaling cascades. Because it is expressed in undifferentiated pulmonary epithelial cells during airway development, it is considered a critical factor in lung branching morphogenesis and epithelial-mesenchymal interaction. After birth, when TGFB's role in development is complete and its expression is markedly decreased in the lung, TGFB remains a mediator of immune cell function and tissue remodeling (2).

In contrast to TGFB, CFTR was immediately identified as a chloride channel because CF patients have an elevated sweat chloride concentration as a primary diagnostic feature. Observations that suggest that CFTR functions as a cell regulator and its presence during development are not yet fully recognized. CFTR appears to play a more indirect, yet a critical role in extracellular signaling pathways during secretory epithelial cell differentiation. Because this role is transient, it is a more difficult concept to understand and demonstrate.

Originally, investigators stated that the CF lung was normal at birth. However, with the extensive genetic screening programs now done in many states, early changes are being found in infants with CF. Airway inflammation is present in infants with CF at 4 weeks of age, before they have clinically apparent disease (7). Independent studies have demonstrated reduced airway function in infants with CF in the absence of clinically recognized lower airway disease (8). Fetuses examined following prenatal diagnosis of CF have been shown to have tracheal epithelial atrophy with cells devoid of cilia (2).

CONCLUSIONS AND FUTURE DIRECTIONS

As shown in the figure, we propose that CFTR is part of a developmental cascade for secretory cells in the lung, intestines, pancreas, and other secretory organs. Disruption of this pathway could occur by either a CFTR mutation, or by other mutations of genes in this cascade as suggested by the work of Groman et al (1). This finding is consistent with the role in CF of other genes in a common secretory cell pathway that includes CFTR as only one of many components.

The lack of CFTR function during development would lead to incomplete differentiation of secretory cells and loss of function. In addition, the failure of secretory cell differentiation leads to a constitutive expression of cytokines that function in development as agents of differentiation. Once the immune system matures postnatally, however, these same cytokines assume a proinflammatory role, leading to chronic inflammation and fibrosis. It remains to be seen how many of the observed paradoxes in CF can be attributed to CFTR's role in developmental regulation. For example, the airway goblet cell that secretes defective glycoconjugates with altered sulfation and sialylation (but has undetectable levels of CFTR mRNA after birth) may simply be immature. Sulfation of lung glycoprotein is characteristic of fetal lung and sialylation and increases during lung differentiation. CF patients have a decrease in surfactant-associated protein A (SPA), but SPA is also a functional marker of differentiation for the lung

Table 2. Comparison of cystic fibrosis transmembrane regulator with developmentally active transforming growth factor-beta-1 (2).

Transforming Growth Factor-Beta-1	Cystic Fibrosis Transmembrane Regulator
Temporal and tissue-specific expression during development	Temporal and tissue-specific expression during development
Expression greater in the fetal lung than in the adult lung	Expression greater in the fetal lung than in the adult lung
Regulates calcium-activated potassium channels	Regulates outwardly rectifying chloride channels, epithelial sodium channels, and calcium-regulated chloride channels
Decreases intracellular pH and modulates Na/H+ exchange activity	Decreases intracellular pH and modulates Na/H+ exchange activity
Modulates protein glycosylation	Modulates protein glycosylation
Knockout animals appear grossly normal at delivery but die within several weeks of birth	Knockout animals appear grossly normal at delivery but die within several weeks of birth

parenchymal type II cell (2). The primary clinical implications of this new paradigm are twofold. First of all, as our data predicted in 1996 (and have, thus far, proven true), gene therapy for CF in adults will have no positive therapeutic effect. However, in utero gene therapy timed to the developmental stage of secretory cell differentiation has the potential to reverse the disease phenotype. Secondly, therapeutic reversal of the disease in adults requires the delineation of the regulatory pathways of the involved secretory epithelium. Pharmacological intervention may be possible once a better understanding of the immature lung in the CF patient occurs.

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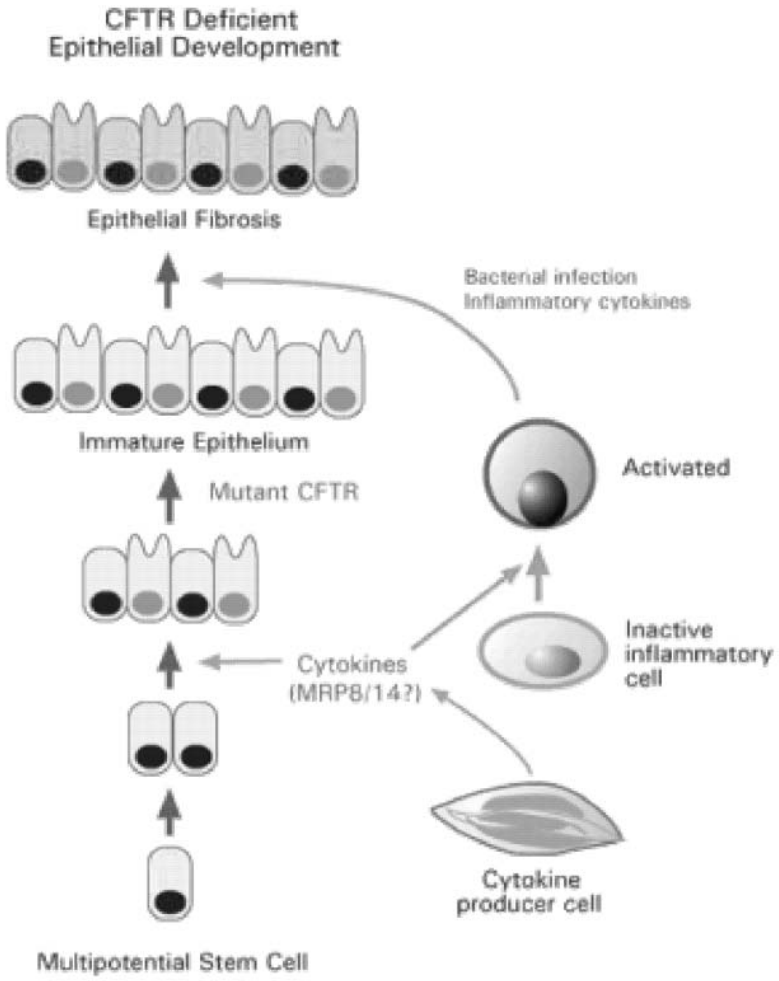

Figure 1.

Cystic fibrosis transmembrane regulator (CFTR) is shown as one member of a developmental cascade required for normal secretory epithelium development. Included in this pathway are other cytokines, possibly MRP8/14(CF antigen). During normal development, the presence of CFTR feedback mechanisms either completely inhibit or at least decrease the expression of these developmentally active cytokines. In contrast, a lack of CFTR function during development would lead to incomplete differentiation of secretory cells. The failure of secretory cell differentiation would lead to the constitutive expression of the developmental cytokines. Once the immune system matures postnatally, these same cytokines would assume a proinflammatory role, leading to chronic inflammation and fibrosis.