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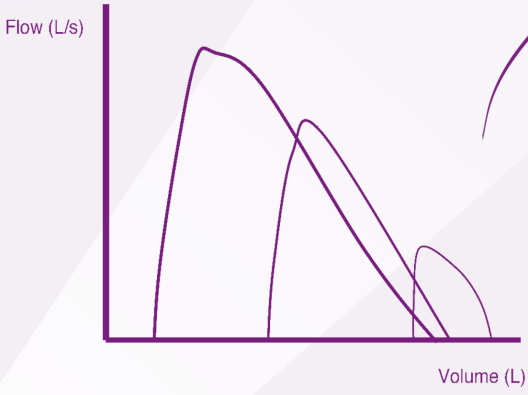
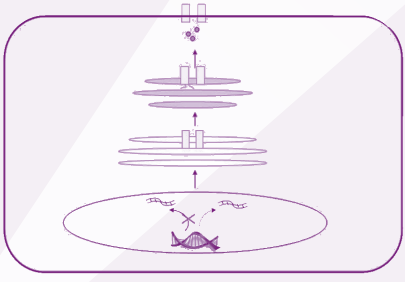
Cystic Fibrosis





Edited by
Julian L. Allen
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LUNG BIOLOGY IN HEALTH AND DISEASE

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Cystic Fibrosis

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To Debby, Eli and Jeremy, whose love, understanding and humor have enabled me to complete this book; to my parents, Beatrice and Emmanuel, who taught me to love music and science...

JLA

To Mary, Oren and Becky, for your encouragement and understanding; to my parents for demonstrating their love of learning...

HBP

To Andrea, Gavriel, Adina and Watson, for sharing this journey; to my parents, Honora and Murray for helping the journey start...

RCR

...and to our patients and their families, who have taught us so much.

Preface

Improvement in the outcome of people with cystic fibrosis (CF) over the past 50 years is truly remarkable. What was once an almost universally fatal childhood genetic disorder has, through advances in research and clinical care, become a chronic illness whereby presently almost half of the people living with CF are adults. The known disease-causing mutations of the cystic fibrosis transmembrane regulator (CFTR) protein gene, which was cloned in 1989, have expanded from just over 150 in 1993 to over 1400 in 2009. Many of the physiologic processes influenced by abnormal CFTR function, from alterations in electrolyte transport to abnormalities of innate airways defense, are now recognized. Nevertheless, CF remains a disease that shortens and alters the quality of the lives of most affected individuals, and much progress remains to be made.

In addition to new insights into the basic mechanisms that cause the CF phenotype, our understanding of the pathogenesis of both pulmonary and extrapulmonary manifestations of the disease have advanced. Concurrently, new techniques have been developed to detect CF lung disease at its earliest stages, well before it is clinically apparent. The confluence of these discoveries has led to the development of new therapeutic agents and approaches for the care of people with CF.

This book attempts to detail recent insights and knowledge of CF pathogenesis, treatment, and health care systems approaches. We hope to make accessible to caregivers an increased understanding of both the molecular basis of CF and its expanding clinical features. We also hope to provide a common knowledge base that will allow the generation of testable hypotheses for future basic, clinical and translational research. We have sought to highlight new challenges for improving care and outcomes; new approaches for diagnosing, assessing, and treating CF; and the "new" clinical manifestations that have resulted from greater longevity of people with CF. We have also endeavored to relate the underlying cellular and molecular pathophysiologies to their relevant clinical phenotypes, and thereby provide the rationale for novel interventions. In this way, we hope this volume gives the reader an idea of how far we have come in the care of people living with CF, how far we still have to go, and some ideas about how we might get there.

A book such as this is not possible without the contributions of many. We would like to thank the editorial staff at Informa Healthcare, especially Joseph Stubenrauch and Aimee Laussen, for their efficiency and especially their willingness to accommodate numerous last-minute changes. And, of course, we would like to thank the authors, whose knowledge and expertise are extraordinary.

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Contents

1
The Genetics of Cystic Fibrosis

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I. The CF Gene

Over recent years there has been a dramatic increase in our understanding of the genetics of cystic fibrosis (CF). In 1949, Lowe et al. postulated that CF must be caused by a defect in a single gene on the basis of the autosomal recessive pattern of inheritance of the disease (1). The identification of the cystic fibrosis transmembrane conductance regulator (CFTR) gene in 1989 by Collins et al. (2–4) created new hope for curative treatment. The CFTR gene is localized on the long arm of chromosome 7 (7q21-34), spanning approximately 190 kb of genomic DNA (5). The gene consists of 27 exons and encodes a mature mRNA transcript of 6.5 kb that is translated into a 1480–amino acid protein.

CFTR is found in various epithelial cell types at the apical surface, including respiratory epithelia and submucosal glands, exocrine pancreas, liver, sweat ducts, and the reproductive tract. In these locations, its main function is to act as a cAMP-mediated chloride channel that regulates the ion and water balance across epithelia (6,7). It has also been reported to be present and to have function in the brain (8), neonatal murine cardiac myocytes (9), erythrocytes (10,11), and macrophages (12), among other cell types.

CFTR is a member of the ATP-binding cassette (ABC) membrane transporter superfamily that includes proteins such as the multiple drug resistance protein (MDR) and bacterial periplasmic permeases. Like other ABC transporters, the CFTR protein contains two homologous halves comprising two membrane-spanning domains, each with six helices, and two nucleotide-binding domains (NBDs) (3). However, unlike other ABC transporters, CFTR has a unique regulatory (R) domain that separates the homologous halves and contains many charged amino acids and consensus sites for phosphorylation by protein kinases (Fig. 1). The two membrane-spanning domains form a low-conductance chloride channel pore (Fig. 1). CFTR is activated by protein kinase A (PKA), and is probably also regulated by protein kinase C (PKC), with phosphorylation occurring at multiple sites located in the R domain. As phosphorylation by PKA is mandatory for channel activity, CFTR channel is considered a "cAMP-activated channel."

II. Incidence

CF remains one of the commonest life-threatening autosomal recessive conditions affecting Caucasians. There are approximately 30,000 affected individuals in the United States, and about 1000 new cases are diagnosed each year. The incidence is 1/2500 to

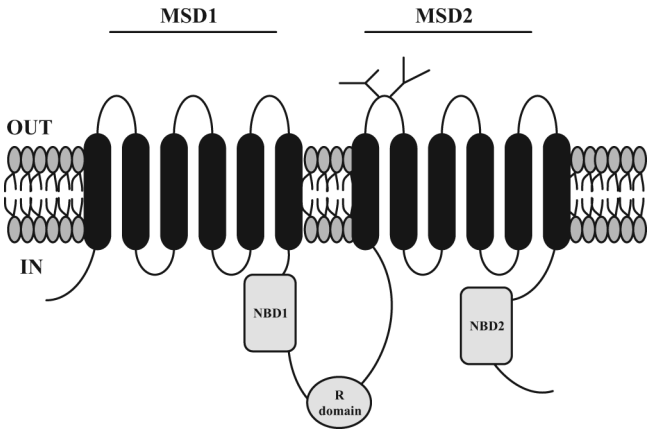

Figure 1 Proposed domain structure of the CFTR protein within the cell membrane. *Abbreviations*: CFTR, cystic fibrosis transmembrane conductance regulator; MSD, membrane-spanning domain; NBD, nucleotide-binding domain; R, regulatory domain.

Table 1 CF Incidence Across the Globe

Country	Incidence (1 case per *x* birth)
Finland	25000
Mexico	8500
Sweden	7300
Poland	6000
North Ireland	5350
Russia	4900
Denmark	4700
Norway	4500
The Netherlands	3650
Spain	3500
Greece	3500
Germany	3300
United States	2835
Czech republic	2833
United Kingdom	2600
Australia	2500
Italy	2438
France	2350
Switzerland	2000
Ireland	1800

Abbreviation: CF, cystic fibrosis.
Source: From Ref. 13.

1/90,000, and varies between populations of different ethnicity (Table 1): in Caucasians the incidence is estimated to be 1/3200; in African-Americans 1/15,000; and in Asian-Americans 1/31,000. Even among northern European Caucasian populations, the incidence varies significantly; the incidences in Ireland and Sweden are 1/1800 and 1/7300 live births, respectively (14). The geographical distribution of CFTR mutations also varies worldwide. These variations are likely to be due to founder effects and subsequent patterns of migration and settlement.

The high prevalence of the CF gene in certain populations has led to speculations that there may be some selective advantage for heterozygotic carriers (15,16). For example, mutations in the CFTR gene are hypothesized to provide increased resistance to infectious diseases, thereby maintaining the mutant alleles at high frequency in selected populations. Pier and colleagues hypothesized that heterozygote carriers of CFTR mutations had increased resistance to infection with intracellular organisms such as *Salmonella* (17), while others hypothesized that carriers have a selective advantage because of resistance to diarrhea-causing enterotoxins elaborated by *Vibrio cholera* and *Escherichia coli* (18).

Cholera toxin (CT) and the heat-labile toxin elaborated by enterotoxic *E. coli* cause irreversible activation of the stimulatory guanine nucleotide–binding protein, G_s, which in turn activates membrane-bound adenylyl cyclase. This greatly elevates cellular cAMP levels and causes subsequent activation of PKA, phosphorylation of R domain of CFTR, and opening of the CFTR channel, which is the predominant cAMP-regulated Cl^- channel in the intestinal and colonic epithelia. The end result of this signaling cascade is massive Cl^- secretion into the intestinal lumen and potentially lethal secretory diarrhea characterized by, in the case of cholera, "rice water stools." The data of Gabriel and colleagues from *cftr* null and heterozygote carrier mice support this selective advantage hypothesis, as well as a central role of CFTR in the Cl^- secretory response of the intestinal epithelia to CT. In response to intraluminal injection of CT, heterozygous CF-carrier mice secreted 50% less volume of fluid into their intestines than wild-type mice under the same conditions. The *cftr*−/− knockout mice had no fluid-secretory response to intraluminal injection of CT. However, this hypothesis was disputed by Hogenauer et al., who measured the in vivo basal (unstimulated) and prostaglandin-stimulated jejunal chloride secretion in normal human subjects, CF heterozygote carriers, and subjects with CF (19); prostaglandins similarly act to increase cellular cAMP. These data indicated that while subjects with CF had essentially no active chloride secretion in response to the prostaglandin secretagogue, individuals who were heterozygous carriers of a CF mutation secreted chloride at the same rate as people without a CF mutation. These data thus contradicted the earlier mouse model data. Furthermore, it is not clear how such resistance to cholera as the selective advantage for heterozygote carriers would have been significantly beneficial in the populations with the highest CFTR mutation carrier frequencies, as cholera is not endemic in those geographic regions.

III. Classes of CFTR Mutations

More than 1500 CF mutations have been reported. Many of these mutations are rare in the population, with only a few affected individuals reported. There are also a number of silent and nonsilent changes in the coding sequence that are not clearly demonstrated to

Table 2 Most Common Mutations Found in the Total U.S. Population

Mutation	Prevalence (%)
DeltaF508	68.6
G542X	2.4
G551D	2.1
W1282X	1.4
N1303K	1.3

Source: From Ref. 13.

induce CFTR dysfunction; such changes are therefore considered CFTR polymorphisms. Thus, the number of true disease-causing mutations is likely fewer [see the CFTR mutations database (20)]. The disease-causing mutations are situated throughout the entire coding region of the gene, and also the promoter and intronic regions, although there are "hot spot" regions where mutations are more common, such as the NBDs, the cytoplasmic loops of the transmembrane domains that are hypothesized to interact with the NBDs (21), and the regulatory (R) domain.

The most common disease-causing CFTR mutation worldwide is ΔF508, which occurs on approximately 70% of mutant CFTR alleles. This absence of phenylalanine at position 508 of the CFTR protein results from an in-frame deletion of three base pairs from exon 10. The ΔF508 mutation results in an abnormal protein that is defective with regards to intracellular processing. This leads to absence of CFTR channels from the membrane and, as a result, absent CFTR function. This is discussed further below. The majority of the other mutations have significantly lower allele frequencies of less than 2% to 3% (Table 2), although this can clearly vary greatly within populations or ethnic groups suggesting founder effects. For example, the W1282X mutation has an allele frequency of 1% to 2% in the North American Population, but is the predominant allele in Israel and in those of Ashkenazi Jewish descent (22). Many of other mutations are unique to a particular individual or family or have been found in only a handful of cases across the world. Given the large and complicated makeup of the CFTR gene and promoter, as well as the large number of described rare mutations found by sequencing the CFTR coding region and targeted areas of intronic and promoter DNA, it is not surprising that such gene sequencing does not always identify two CFTR mutations in people with CF. Using present commercial techniques, up to 1% of people with a clinical diagnosis of CF may not have two identifiable CFTR mutations.

It has become convenient to classify the large number of described CFTR mutations into six different groups according to the mechanism by which they disrupt CFTR Cl^- transport function (Fig. 2) (23). However, for the vast majority of CFTR mutations, especially rarer mutations, mechanistic data on which to properly classify such mutations according to this scheme is lacking. Furthermore, there are a number of mutations that either have mechanistic defects that overlap two classes or frankly defy classification according to this scheme. Nevertheless, we briefly discuss this classification scheme here, as it has been clearly useful in assisting clinicians and investigators in thinking about genotype-phenotype correlation in people with CF- or CFTR-related disorders, and in guiding the development of novel, mutation-directed therapies for CF (discussed in Chapter 25 by Hoover and Clancy).

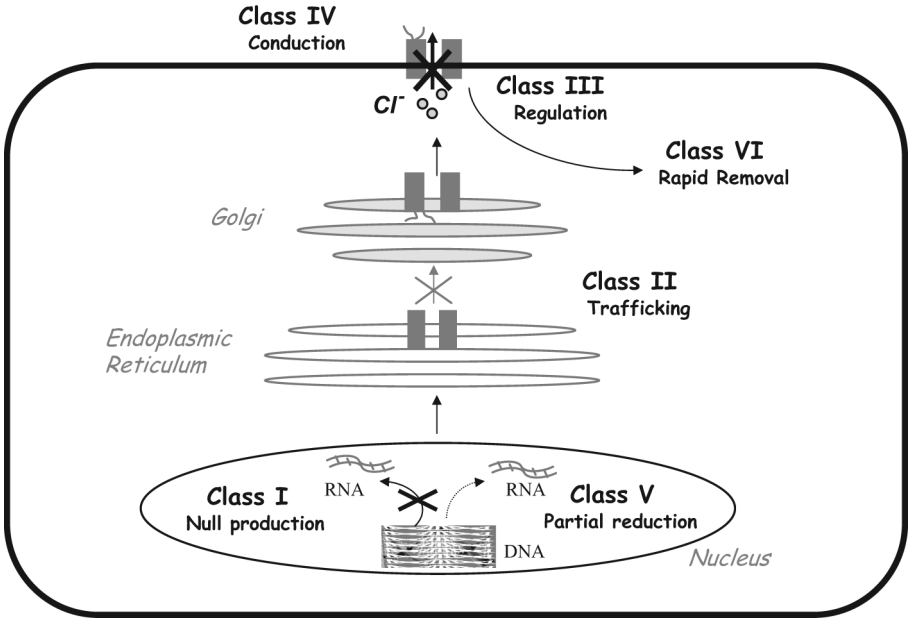

Figure 2 Classes of mutant CFTR. *Abbreviation*: CFTR, cystic fibrosis transmembrane conductance regulator.

Class I mutations abolish protein production and result in complete loss of CFTR protein, and therefore CFTR function. About half of all mutations in CFTR (encompassing gene deletions, exon skipping due to aberrant mRNA splicing, and single nucleotide or smaller deletions leading to reading frame shifts) are thought to fall into this class. Class I also includes mutations that generate premature stop codons, including G542X and W1282X, that lead to early termination of protein translation and rapid mRNA degradation (24). Combined, these "X" mutations have an overall allele frequency of approximately 10%, and, as described in Chapter 25, may be targets for therapies that allow the ribosome to "read through" or suppress these premature termination codons.

Class II CFTR mutations are characterized by aberrant intracellular trafficking of the CFTR protein, and therefore the absence of the functional protein from the apical membrane of epithelia. Mutations of this class include the common ΔF508, and N1303K. In the case of ΔF508, the mutant CFTR is recognized by the cell quality control mechanism and subsequently degraded. Interestingly, ΔF508 retains the ability to transport Cl^- across intracellular membranes (25), and a number of physical and chemical maneuvers (26), including reduced temperature incubation (27), allow it to reach the apical membrane. However, both ΔF508 (28) and N1303K (29) appear to have abnormal functions even when at the plasma membrane. This suggests that these mutations make CFTR dysfunctional by multiple mechanisms, and that these mutations may, in fact, have characteristics of more than one CFTR mutation class.

Class III mutations disrupt activation and regulation of mutant CFTRs, which are appropriately localized at the plasma membrane. Thus, biosynthesis, trafficking, and

processing are undisturbed, but the channel may be defective with respect to phosphorylation by PKA or the subsequent regulation of channel opening. This class of dysfunction tends to result from missense mutations in key areas of the protein that are important for regulation of CFTR function (30). The most common mutation of this class is G551D, which has an allele frequency of approximately 2%. G551D is a mutation in CFTR's first nucleotide-binding fold (NBD-1), which results in a very low channel open probability. This leads to essentially absent CFTR function despite G551D-CFTR's appropriate localization at the apical membrane of epithelia.

Class IV CFTR mutants are defined by aberrant or reduced chloride conduction. These mutants tend to result from more conservative missense mutations of CFTR, and retain normal intracellular location of the mutant protein at the apical epithelial membrane (31). The most common of these class IV mutations is R117H, which is the substitution of a slightly less strongly positively charged histidine for a strongly positively charged arginine residue.

Class V mutations reduce the amount of normal CFTR protein in the cell and at the apical membrane by decreasing, but not eliminating, protein production. However, in general, the CFTR protein that is produced by mutations of this class functions normally. Such effects may result from mutations in the promoter or by inefficient mRNA splicing. One example of a class V mutation is the 3849 + 10 kb C→T mutation found in intron 19, which reduces the splicing efficiency of the CFTR mRNA to approximately 8% of normal (32). Another intronic mutation, 2789 + 5 kb G→A reduces mRNA splicing efficiency to approximately 4% by altering the splice donor site of exon 14b (33).

Class VI mutations, like class V mutations, reduce the amount of functional CFTR protein at the apical membrane. However, in contrast to class V mutations that decrease CFTR production, class VI mutations cause an increased rate of CFTR's removal from the apical plasma membrane. Mutations leading to this type of CFTR defect are uncommon and include N287Y (34) as well as mutations that delete the carboxyl terminus of the CFTR protein.

IV. Genotype/Phenotype Correlation

In general, mutations of classes I, II, and III are associated with absent CFTR function. Thus, any person with CF who is homozygous for a mutation of one of these classes, or who is compound heterozygous for any combination of class I, II, or III mutations, is expected to have an overall absence of CFTR function. This typically results in a "severe" or "classic" CF phenotype including exocrine pancreatic insufficiency. This phenotype is present in 85% to 90% of people with CF. Further attempts to delineate genotype/phenotype correlations in this group of people with CF have been less successful, perhaps because almost 50% of people with CF are ΔF508-homozygotes, and additional approximately one-third are ΔF508 compound heterozygotes.

On the other hand, even among the people with CF who are ΔF508-homozygous, there is tremendous variability in phenotype and clinical course. This has led to the hypothesis and recent demonstration that polymorphisms in other genes, such as transforming growth factor β (TGF-β), are associated with significantly different clinical CF phenotypes (35). The topic of genes that modify the CF phenotype is discussed in more depth elsewhere in Chapter 6 by Drumm.

The presence of class IV and V CFTR mutations are associated with significant residual CFTR function, even when present with a second allele where function is absent (i.e., a class I, II, or III mutation). This residual CFTR function of the class IV or V allele usually manifests clinically as phenotypically milder CF, often with exocrine pancreatic sufficiency and less sinopulmonary symptomatology. This milder phenotype occurs in the remaining 10% to 15% of people with CF.

Interestingly, the amount of residual function of a class IV or V mutant CFTR, such as R117H, and subsequently clinical phenotype can be significantly modified by polymorphisms that influence how much mRNA encoding R117H, and therefore how much R117H protein is made. One well-studied example of this is exon 8-9 splicing. Failure to correctly splice exon 8 to exon 9 results in a nonfunctional CFTR, and the efficiency and fidelity of this reaction is influenced by a polythymidine sequence within intron 8 preceding the exon 9 splice acceptor site (36). This polythymidine tract is polymorphic with sequences of 5, 7, or 9 thymidines (5T, 7T, or 9T, respectively), and the efficiency of exon 8-9 splicing is directly proportional to the length of the thymidine sequences. The 9T variant allows the greatest proportion of normal exon 8-9 splicing and functional CFTR production, while the 5T variant is associated with the highest level of mRNA missplicing and nonfunctional CFTR protein production (36). The commonest polymorphism is the 7T variant, which has a splicing efficiency intermediate to that of 5T and 9T. The ΔF508 mutation occurs exclusively associated with the 9T variant, while other class I, II, or III mutations are more often found in *cis* with 9T than is wild-type CFTR (37). These data suggest that evolution has either sensed that more CFTR function is needed when CFTR is mutant, or that CFTR, when present, should have limited expression.

In the case of R117H, this intron 8 polymorphism can clearly modulate phenotype. For a person with one R117H allele and a second nonfunctional allele of class I, II, or III, such as W1282X, ΔF508, or G551D, those with 5T in *cis* with R117H typically have more clinical symptoms of CF, including sweat chloride elevation, than do people with 7T in *cis* with R117H, who may even have normal or borderline abnormal sweat tests. Those with 9T in *cis* with R117H can, in fact, have minimal, if any, outward symptoms of classical or even pancreatic sufficient CF (36,38,39).

Interestingly, the penetrance of the 5T polymorphism in men with congenital bilateral absence of the vas deferens (CBAVD) can be significantly influenced by a number of TG repeats directly adjacent to and upstream of 5T tract. In men with CBAVD who had a severe CFTR mutation (class I, II, or III) on one allele and a normal CFTR in *cis* with 5T on the other allele, a greater number of TG repeats, 12 or 13, adjacent to 5T were associated with a higher likelihood of CBAVD than if 11 TG repeats were present (40). This is the likely result of a greater number of TG repeats in *cis* with 5T, causing a further increase in exon 9 skipping during mRNA splicing (41). This would further decrease production of functional CFTR protein. This TG repeat polymorphism may also influence the penetrance of the R117H mutation. These issues are discussed further in Chapter 8 by Ooi, Tullis and Durie.

The phenotypic implications of class VI mutations are less well defined. N287Y (34) is associated with a pancreatic sufficient phenotype according to the CFTR mutation database (20). In contrast, mutations that delete the carboxyl terminus of CFTR may be associated with a greater impairment of CFTR function and pancreatic insufficiency.

V. Heterozygote Carriers of CFTR Mutations

It has been generally accepted that heterozygote carriers of CFTR mutations were "healthy" and essentially unaffected by having one dysfunctional CFTR allele. Recent data have challenged this belief and have suggested that there is an increased risk of clinically apparent disease in heterozygous carriers of CFTR mutations in tissues where CFTR has important epithelial function. For example, people with chronic rhinosinusitis have an increased frequency of being heterozygous carriers of CFTR mutations than does the general population (42). Similarly, heterozygote carriers of the ΔF508 mutation have an increased prevalence of asthma compared with the general population (43), and there is a higher prevalence of CFTR missense mutations in those with asthma than in the general population (44).

Nonrespiratory epithelia also appear at risk in heterozygote CFTR mutation carriers. There are increased incidences of CFTR mutations found in people with chronic idiopathic pancreatitis (45), primary sclerosing cholangitis (46), and CAVBD (47). These recently recognized CFTR-related disorders will be explored in more depth in Chapter 8.

VI. Summary

CF is a monogenic, autosomal recessive condition that results from the absence of the CFTR. Its classic presentation is associated with essentially absent CFTR function. Non-CFTR genetic loci can significantly modulate the clinical manifestations of CF. Recent data also suggest that relatively small modulations of CFTR function may significantly alter the CF phenotype. Our understanding of a potential selective advantage for heterozygote carriers that has allowed persistence of CFTR mutations in the population is limited. This is especially apparent since heterozygote carriers of CFTR mutations also appear at an increased risk for clinical manifestations related to dysfunction of their CFTR-expressing epithelia.

Acknowledgment

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2
Ion Transport

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I. Introduction

Cystic fibrosis (CF) is a disease of abnormal ion transport. Specifically, abnormalities in the expression and function of the cystic fibrosis transmembrane conductance regulator (CFTR) result in abnormal salt and water transport across epithelial surfaces in the gastrointestinal and hepatobiliary systems, respiratory tract, reproductive system, and sweat glands. With the exception of the sweat glands, abnormal salt and water transport eventuate in end-organ damage causing significant morbidity and severely shortening life span.

The connection between abnormally high salt concentration in sweat and fatal disease has been recognized since the Middle Ages. If a child tasted salty when kissed on the forehead, the child was "bewitched or fascinated and was feared to die soon" (1). This saying is assumed to reflect the existence and recognition of CF (2). We now know much more about the child who tastes salty as well as the genetic and molecular mechanisms underlying CF. Through this understanding and with advances in nutritional and pulmonary therapies, mean predicted survival in CF is now more than 37 years. In this chapter, we review the evolution of our knowledge of ion transport defects in CF, describe the ion channel properties of normal CFTR, discuss how changes in the amino acid sequence of CFTR result in channel dysfunction, and review how loss of CFTR function results in end-organ damage, especially in the lungs.

II. Establishing A Role for Ion Transport in CF

The first description of CF as a pathological, genetic entity in the United States was published in 1938 by Dorothy Andersen, MD, a pathologist at The Babies' and Children's Hospital of Columbia University in New York City. This paper entitled "Cystic fibrosis of the pancreas and its relation to celiac disease (3)" firmly established CF of the pancreas as a diagnosis separate and apart from celiac disease. It was not until more than a decade later, however, that the connection was made between salt transport and CF of the pancreas. In 1951, Kessler and Andersen reported on 12 children admitted to Babies' Hospital with heat prostration who were in relatively good health before a heat wave, and who presented acutely with vomiting and signs of shock without evidence of infection. All of these children, except for one who died, responded quickly to rehydration (4). In patients for whom laboratory data were available, serum electrolyte analyses showed low Cl^- and high HCO_3^- concentrations that were reversed with

therapy. These findings supported an etiological hypothesis that "fibrocystic disease is associated with widespread abnormality of epithelial glands (4)."

Following these observations, Paul di Sant'Agnese, MD, also at Columbia University, prospectively studied sweat electrolyte levels in 43 patients and 50 controls. His results demonstrated that Na^+, K^+, and Cl^- all were elevated in the sweat of CF patients, with sweat Na^+ and Cl^- levels being markedly elevated (5). The authors also demonstrated that the elevated sweat Na^+ and Cl^- levels were not the secondary result of pancreatic dysfunction, pulmonary disease, adrenal dysfunction, or renal disease, and concluded that the increased susceptibility to dehydration in CF was due to increased salt loss from sweat glands. These findings led directly to the development of the sweat test as a diagnostic test for CF (6).

III. Finding The Gene

The paper by di Sant'Agnese and colleagues included a statement that CF was a disease of altered ion transport, as opposed to one of abnormal mucus composition or secretion, because, although sweat glands had normal morphology, their abnormal function distinguished patients with CF from others (5). Nonetheless, there remained the clinical observations that end-organ damage in the pancreas and lung was characterized by thick, sticky mucus. The question therefore arose of how to rectify these two apparently disparate observations. Over the last five decades, this question has inspired more than 30,000 papers indexed in PubMed.

By the early 1980s, observations in the sweat gland, pancreas, and respiratory tract began to suggest that CF was, at its essence, a disease of altered anion transport. Multiple studies confirmed the findings of di Sant'Agnese that sweat electrolyte concentrations were abnormal in CF. In separate studies using different techniques, Quinton (7) and later Fromter (8) concluded that CF sweat glands had decreased ductal Cl^- permeability and reduced secretion in response to adrenergic stimulation. Similarly, studies of pancreatic HCO_3^- secretion in CF patients concluded that abnormal pancreatic secretion in CF could be attributed at least in part to altered Cl^- secretion (9). Knowles and colleagues at the University of North Carolina reported that the electrical potential across the nasal epithelia of CF patients was more electronegative than controls and did not respond appropriately to adrenergic stimulation (10). It seemed, then, that the basic defect in three different organ systems could be attributed to altered Cl^- permeability.

Armed with this knowledge, CF researchers began to search for the affected gene. In 1985, two laboratories using different markers for linkage analysis localized the gene to the long arm of chromosome 7 (11,12). In 1989, Tsui and colleagues discovered the gene responsible for CF (13) and found that in the majority of patients the gene was missing three nucleotides that resulted in the in-frame deletion of a phenylalanine residue at position 508 of the polypeptide chain (ΔF508) (14). They designated the protein as the CFTR (14). In doing so, the group recognized that if CFTR was not itself a Cl^- channel, then the protein would almost certainly function as a regulator of Cl^- channel activity.

IV. Normal CFTR

Even before the CFTR gene was cloned, it was known that cAMP-stimulated Cl^- secretion was defective in CF epithelial cells (15). Shortly after the CFTR gene was identified, data emerged that this defective cAMP-mediated Cl^- secretion could be corrected by expression of normal CFTR, but not by expression of ΔF508 CFTR. These data supported the hypothesis that CFTR was a Cl^- channel, but still left open the

possibility that it was functioning as a positive regulator of another Cl^- channel (16,17). In 1991, Anderson and colleagues at the University of Iowa expressed recombinant CFTR in three different cell lines, and conferred on those cells a cAMP-activated Cl^- conductance that was not found in cells expressing ΔF508 CFTR (18). These results were independently confirmed in other cell lines (19,20). Demonstration that mutating specific amino acids in CFTR altered the anion selectivity of the ion permeation pathway conferred on cells in which CFTR was heterologously expressed also strongly suggested that CFTR was a Cl^- channel (21). Finally, Bear and colleagues purified the CFTR protein, expressed it in isolated planar lipid bilayers, and demonstrated that it had ion permeation and gating (opening and closing activity) properties identical to those of CFTR heterologously expressed in cell culture (22).

When studied by standard electrophysiological techniques in either native or heterologous systems, CFTR has a characteristic biophysical profile. It is an anion-selective channel with a single channel Cl^- conductance of 6 to 10 picosiemens (pS) in approximately 120 mM Cl^- and a permeability selectivity sequence $Br^- \geq Cl^- > I^- > F^-$ (23,24). CFTR can also conduct HCO_3^- (25,26). When studied by patch clamp electrophysiology in symmetrical Cl^--containing solutions, CFTR channels demonstrate a linear current-voltage relationship (Fig. 1) (18). The opening of the anion permeation pathway in CFTR requires phosphorylation of the channel, particularly by cAMP-dependent protein kinase A (27), as well as the presence of ATP (28).

CFTR is a unique member of the ATP-binding cassette family of transporters (ABC transporters), which ordinarily use energy from ATP hydrolysis to pump substrates actively through the protein and across the membrane (29). CFTR has seven domains: cytoplasmic amino and carboxyl termini, two membrane-spanning domains

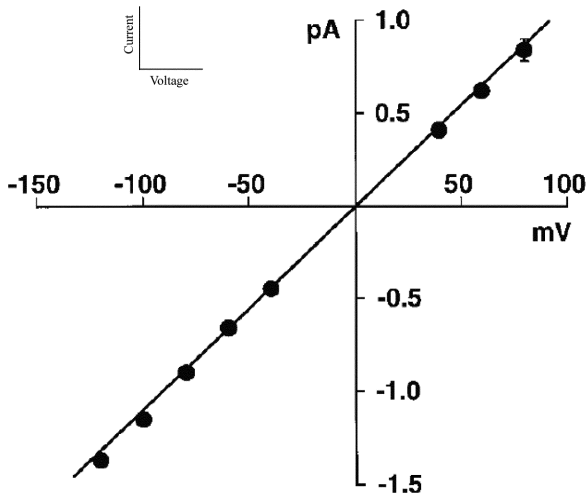

Figure 1 Current-voltage (*I-V*) curve from an excised, inside-out patch containing a single wild-type CFTR channel bathed in symmetrical Cl^- solutions at 35°C. Note the linear relationship between current and voltage. The calculated single channel conductance is 6 to 10 pS. *Source*: From Ref. 24.

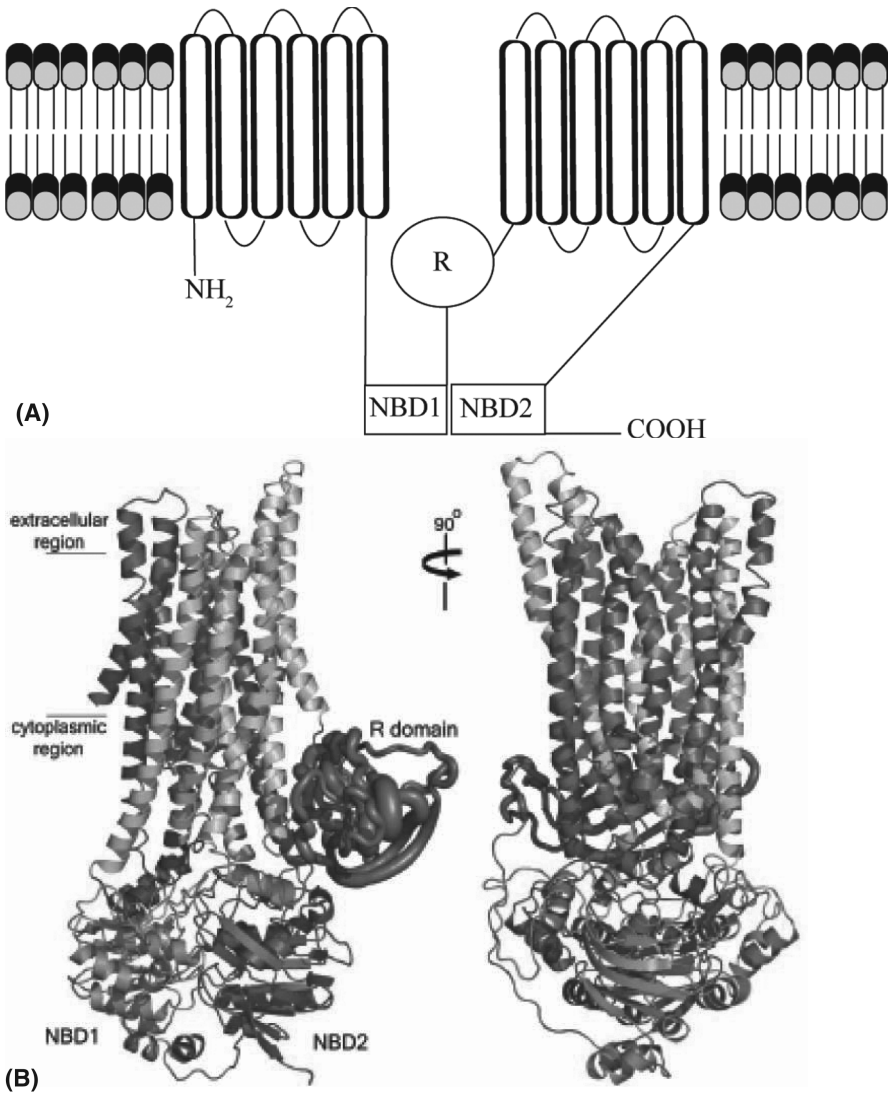

Figure 2 (**A**) Commonly accepted model of CFTR structure based on its amino acid sequence. (**B**) Recently published three-dimensional structure of CFTR based on homology modeling. *Source*: Part A adapted from Ref. 14 and part B from Ref. 30.

that each contain six membrane-spanning segments, two nucleotide binding domains (NBD1 and NBD2) and an R, or regulatory, domain (14). A high-resolution structure of full-length CFTR has not yet been determined, but homology modeling based on crystal structures of bacterial ABC transporters has provided clues about CFTR's possible three-dimensional architecture in cell membranes (Fig. 2) (30). In addition, functional

studies have revealed how each domain plays a role in the function or regulation of the channel. The putative 12 transmembrane helices provide the anion permeation pathway and contain the gate that controls transmembrane anion flux. The two NBDs of CFTR bind and/or hydrolyze ATP to modulate channel activity in a manner that is not yet completely determined, but likely involves dimerization of the two NBDs (31). Nucleotide binding and/or hydrolysis induces conformational changes of the NBDs that are somehow communicated to the channel gate in the transmembrane domain that result in its opening and closing. This communication may be mediated by extensions of the transmembrane helices that interact with the NBDs. The R-domain of CFTR, unique among ABC transporter family members, is rich in consensus phosphorylation sites, mainly for protein kinases A and C (32). However, other kinases can also phosphorylate CFTR (33). Phosphorylation of CFTR is necessary for CFTR activation (28), and CFTR channels are deactivated upon dephosphorylation carried out by protein phosphatases (34,35). The amino and carboxyl terminal regions have specific amino acid residues that allow CFTR to bind to intracellular proteins (36). For example, the carboxyl terminus interacts with the scaffolding protein NHERF-1 (37), which modulates channel gating (38) and enables CFTR to interact with other proteins (39). In addition to CFTR channel activity regulation by nucleotide binding, phosphorylation, and protein interactions, the amount of CFTR in the plasma membrane is also regulated by its trafficking and recycling in and out of the apical membrane of epithelial cells (40,41).

V. Abnormal CFTR and Tissue-Specific Ion Transport Abnormalities

As discussed elsewhere in this volume, CF is an autosomal recessive disorder, meaning that a person must have two abnormal CFTR genes to manifest the abnormal epithelial ion transport characteristic of the disease. A patient's clinical phenotype will usually reflect full loss of CFTR ion transport function, or if there is residual ion transport function afforded by one of the mutant alleles. For example, patients who have a single R117H CFTR allele have less severe reduction of plasma membrane anion permeability (42,43) and generally have milder disease than patients where CFTR function is absent.

The pathophysiology of end-organ damage in CF patients differs from one organ system to the next; however, the basic defect—a lack of apical plasma membrane Cl^- and HCO_3^- permeability in epithelial cells—remains the same. Although CF pancreatic and pulmonary disease are characterized by luminal obstruction and fibrotic parenchyma, the sweat gland is unique in that it does not demonstrate any macroscopic pathological defects such as luminal obstruction or scarring. This may be because the sweat gland does not secrete significant amounts of protein or mucus that need to be flushed from its lumen, as is the case in other tissues affected in CF. That the physiology of the sweat gland is abnormal in CF despite its not being a mucus-secreting epithelium strongly supports the notion that the basic defect in CF is confined to abnormal ion transport rather than extending to basic abnormalities in mucus production or secretion.

A. The Sweat Gland

The human sweat gland comprises a coiled secretory acinus connected to an absorptive duct that empties at the surface of the skin. In the secretory acinus, fluid isotonic to plasma is derived primarily by cholinergic-stimulated, CFTR-independent salt secretion.

As isotonic fluid moves up the sweat duct, NaCl is absorbed in a CFTR-dependent manner so that in normal individuals sweat becomes hypotonic to plasma at the skin surface; this allows sweat to easily evaporate from the skin and effect cooling. The sweat gland is unique among epithelia in that CFTR is detected at both apical and basolateral membranes of cells lining the absorptive duct (reviewed in Ref. 2). In CF, lack of Cl^- permeability in the sweat duct impairs NaCl reabsorption, causing excretion of sweat with high salt content (5). This salty sweat evaporates less readily and leaves small salt crystal deposits on the skin.

In addition to cholinergically mediated secretion, sweat secretion can be stimulated through adrenergic pathways (44). The rate at which sweat is produced after β-adrenergic stimulation is related to the amount of functional CFTR present in the sweat duct. Therefore, in response to β-adrenergic stimulation, patients without CFTR mutations have higher sweat rates, carriers of one CFTR mutation have intermediate rates of sweat production, and CF patients have virtually no sweat production (45,46). This has led some investigators to propose the use of sweat rates as an end point for clinical trials of pharmaceutical agents aimed at correcting the underlying molecular defect in CF, the lack of functional CFTR (47).

B. The Pancreas

The pancreas consists of exocrine and endocrine cellular components. Although both can be affected in CF, this section will focus only on the exocrine pancreas because it is primarily affected by the ion transport defect in CF, whereas the endocrine dysfunction is, at least in part, secondary to pancreatic tissue damage. The exocrine pancreas is similar to the sweat duct in that it consists of secretory acini connected to ducts where the ionic composition of acinar secretions is modified. In response to stimuli that elevate intracellular Ca^{2+}, pancreatic acinar cells release zymogen granules to secrete a digestive enzyme-rich fluid into the lumen of the acinus. This protein-rich fluid is hydrated and alkalinized in the duct lumen by CFTR-dependent Cl^-, HCO_3^-, and fluid secretion that flushes the contents into the intestinal lumen.

In CF, the ability of the pancreatic ducts to secrete fluid is severely impaired because of absence of a functional interaction between CFTR and an apical Cl^-/HCO_3^- exchanger (48–50). In this model, CFTR serves at the apical membrane of pancreatic duct cells both as a conductive pathway for Cl^- and as a critical regulator of Cl^-/HCO_3^- exchanger activity. As a result of absent ductal HCO_3^- and fluid secretion, acinar secretions become dehydrated and trapped in pancreatic ducts. This blocks secretion of both fluid and digestive enzymes into the intestine, and allows the digestive enzymes to act on pancreatic parenchyma causing inflammation and scarring that eventuate in destruction and fibrosis of the exocrine pancreas.

C. The Intestine

The intestinal epithelium is histologically divided into villi and crypts and glands. CFTR is primarily expressed in intestinal crypts (51) where it promotes Cl^- and fluid secretion that hydrates intestinal mucins produced in the glands. The primary mode of fluid secretion in the small intestine is the activation of CFTR at the apical membrane of intestinal epithelial cells and movement of Cl^- down its electrochemical gradient into the lumen of the intestine. The opening of CFTR at the apical membrane and subsequent secretion of Cl^- into the lumen of the intestine is accompanied by activation of the loop-diuretic (e.g., furosemide)

sensitive Na^+-K^+-$2Cl^-$ cotransporter on the basolateral membrane that provides a mechanism for Cl^- entry into the cell (52). Na^+ moves across the epithelium through paracellular pathways to maintain electroneutrality, and water follows osmotically.

In CF, the absence of functional CFTR results in lack of Cl^- secretion and associated fluid secretion so that intestinal mucus and stool are poorly hydrated. Histologically, retained mucins in intestinal crypts seen in pathology specimens are virtually pathognomonic for CF (53). Poorly hydrated intestinal mucus and stool can become trapped in the intestine causing partial or complete luminal obstruction. In the newborn, this process manifests as failure to pass meconium, known as meconium ileus. In the infant and older child, this manifests as inspissations of mucus and stool in the distal small intestine known as distal intestinal obstructive syndrome or DIOS.

D. The Liver

Severe liver disease is the most common nonpulmonary cause of mortality in CF. Ion transport abnormalities in the liver result in abnormal salt and water transport resulting in blockage of biliary ducts, and in most patients, cirrhosis of the liver (54). Obstruction of biliary ducts causes periportal inflammation and fibrosis, which occurs in approximately 30% of CF patients (55). In a subset of these patients, liver damage progresses to become multilobular cirrhosis and may result in portal hypertension. This topic is discussed in detail in chapter 18.

E. The Reproductive Tract

More than 90% of males with CF have congenital bilateral absence of the vas deferens (CBAVD). Furthermore, 1% to 2% of non-CF male infertility is due to CBAVD, and these patients have a higher incidence of CFTR mutations than do patients without CBAVD (56). Therefore, the link between CFTR and CBAVD is firmly established. The role of abnormal ion transport in the pathophysiology of CBAVD is less clear. It may be that absence of normal salt and water transport in the vas deferens leads to blockage of the lumen during development of the vas deferens causing subsequent involution.

F. The Lungs

The mean age of predicted survival in CF has increased from less than a year in the 1950s to almost 40 years today. When CF patients died in infancy, it was largely the result of malnutrition or heat prostration, though most showed signs of lung disease at autopsy (3). As patients have survived longer, lung disease has emerged as by far the most common cause of morbidity and mortality in CF. The end result of CFTR dysfunction in the lung is often described as a vicious cycle of infection, inflammation, and tissue damage resulting from obstruction of the airways by thick, sticky mucus.

What is the entry point into the vicious cycle? There are a number of hypotheses that try to answer this question. One well-accepted hypothesis states that lack of CFTR function in the apical membrane of airway surface epithelial cells results in hyperabsorption of NaCl from the airway surface liquid (ASL) and subsequent dehydration of airway lining fluid and mucus. This allows airway mucus to appose airway epithelial cells, which in turn flattens cilia and renders them unable to beat. Accordingly, airway mucus becomes trapped and inhaled bacteria are not cleared appropriately.

This hypothesis encompasses two, not mutually distinct, hypotheses. The first of these hypotheses is that the lack of CFTR results in overactivation of the cellular Na^+ absorption pathway via the epithelial sodium channel (ENaC). This overactive Na^+ absorption drives Cl^- absorption through the paracellular pathway to maintain electroneutrality. The second hypothesis includes a normal role for CFTR as a secretory pathway for Cl^-; this Cl^- secretion drives fluid secretion as is described earlier for the intestine. Both of these hypotheses have substantial in vitro and in vivo scientific support. In vivo evidence includes the elevated sensitivity to the ENaC-blocker amiloride in nasal potential difference measurements in CF epithelia compared to control epithelia (10,57) and the finding that overexpression of ENaC in mice caused CF-like airway disease (58).

To study the effect of CFTR dysfunction on airways ion transport physiology in vitro, researchers have relied on airway epithelial cells grown and differentiated on permeable supports and exposed to air on their mucosal surface. First described using guinea pig cells (59) and later adapted for human cells (60), this model system recapitulates many of the normal functions of the airway epithelium (Fig. 3). In vitro measurements of ASL height in these well-differentiated, polarized cells strongly suggest that ASL height is reduced in cells from CF donors compared with normals (61). This model system has also been used in attempts to dissect the mechanisms by which Na^+ absorption is increased in CF epithelial cells. One hypothesis is that absence of CFTR leads to elevated concentrations of proteases in the ASL that activate ENaC (62,63). Another hypothesis suggests there is a reciprocal relationship between CFTR

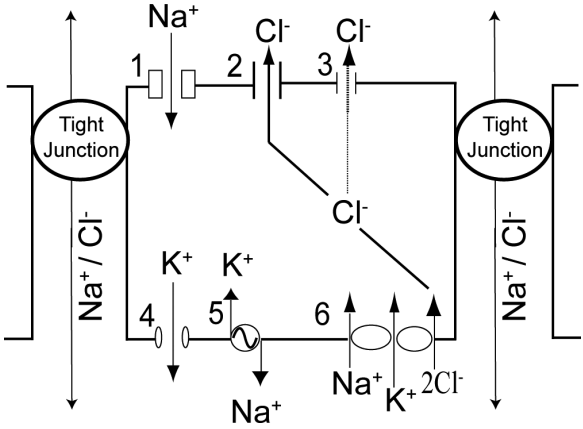

Figure 3 Simplified model of epithelial sodium and chloride transport. For Na^+ absorption, Na^+ enters from the lumen through the epithelial sodium channel (ENaC, #1) and is pumped out of the cell by the Na^+/K^+-ATPase (#5), which maintains ionic gradients at the expense of ATP hydrolysis. For Cl^- secretion, Cl^- enters the cell from the blood on the Na^+-K^+-$2Cl^-$ cotransporter (NKCC-1, #6) and can exit the cell to the lumen through two separate channels. cAMP-mediated secretion occurs via CFTR (#2), which is the predominant Cl^- conductance in most epithelia. Ca^{2+}-activated Cl^- secretion occurs via the Ca^{2+}-activated Cl^- channel (TMEM16A, #3). Both Na^+ absorption and Cl^- secretion are dependent on apical membrane hyperpolarization by basolateral K^+ channels (#4), which serve as a shunt pathway for K^+ that enters the cell on the Na^+/K^+-ATPase. Electroneutrality is maintained by paracellular movements of Na^+ and Cl^-.

and ENaC at the plasma membrane (64). On the other hand, activation of an alternative apical membrane Cl^- channel in the absence of CFTR can stabilize ASL volume (65). These data suggest that CFTR may normally play a role in counteracting Na^+-driven fluid absorption by providing a mechanism for Cl^--mediated fluid secretion. Lack of this counterbalance results in excessive salt and water absorption.

The salt hyper-absorption hypotheses address the function of surface epithelial cells in removal of salt and water from the airway surface, but they do not address the possible roles of submucosal glands in the pathophysiology of CF. Anatomically, submucosal glands are found in highest density in the trachea and bronchi, but they can be found throughout the conducting airways, including small bronchi. Submucosal glands may secrete the majority of mucus and fluid that make up the ASL. CFTR is highly expressed in the serous cells of submucosal gland acini, even more highly than in the airway epithelia (66), where it plays a role in Cl^-, HCO_3^-, and fluid secretion (67). Some evidence suggests that hyposecretion from submucosal glands in CF is a proximal cause of lung disease, and that this lack of secretion is due to absence of Cl^- and HCO_3^- secretion (68). Hyposecretion from submucosal glands could also contribute to an imbalance of proteases and antiproteases in the CF airway (68), which could impinge on the activity of ENaC, as discussed earlier.

A role for altered HCO_3^- secretion by airways epithelial cells and submucosal glands has been hypothesized in the pathophysiology of CF lung disease. In vitro, lack of CFTR-mediated HCO_3^- secretion by cultured bronchial epithelial cells results in defective alkalinization after acid challenge (69). In airway submucosal glands ex vivo, inhibition of HCO_3^- transport results in decreased fluid secretion (67). Furthermore, submucosal glands from CF patients secrete a more acidic fluid (70). Both pH and concentration may affect the manner in which macromolecules such as mucins and proteases behave. Taken together, these data suggest that altered HCO_3^- secretion may play a significant role in the pathogenesis of airways disease in CF.

VI. Summary

In summary, our understanding of CF pathophysiology has progressed from folklore to scientifically based understanding of organ and tissue pathophysiology to subcellular and molecular identification of the basic cellular defects. CF is a disease of altered ion transport resulting from abnormal expression and function of CFTR, an anion channel found in apical membrane of many epithelia. In each affected organ, absence of CFTR is the proximate cause of disease. In some cases, such as the sweat gland, we understand completely how absence of CFTR causes altered organ function. In other cases, such as the lungs, there are multiple effects of CFTR absence that may impact on the disease, and questions remain regarding the underlying molecular mechanisms. Despite these questions, our understanding of how basic defects in CFTR lead to end-organ damage is much greater, and patients have benefited directly.

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3
Mucus Abnormalities and Ciliary Dysfunction

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I. Introduction to Mucus and Mucociliary Clearance

The unavoidable development of chronic bacterial lung infections in cystic fibrosis (CF) results from a significant defect in lung host defenses. Because adaptive defenses appear to be intact in patients with CF, a search for mechanisms to explain impaired innate defense of the lung has been vigorously pursued. The mucus clearance apparatus plays a key role in lung defense, thus it is not surprising that it has become a prime focus of study and therapeutic development for CF.

Effective mucus clearance depends on coordinated ciliary motion and the formation of an airway surface liquid (ASL) layer that is capable of supporting mucus transport via cilia-driven and cough clearance. Traditionally, the ASL has been depicted as the aggregate of two distinct phases: a superficial mucus layer and an aqueous periciliary layer (PCL) that approximates the height of extended cilia. In this paradigm, mucus serves to entrap foreign particles and pathogens, and dissolve noxious gases. The PCL, in turn, supports the transport of mucus out of the lung by providing a low-viscosity environment for cilia beating (1) (Fig. 1). In this chapter, we will review our current understanding of the structure and function of the ASL, with particular attention to changes that may result in impaired host defense in CF.

II. The Mucus Layer

Secreted mucus is a nonhomogeneous, adhesive, viscoelastic gel composed of water, carbohydrates, proteins, and lipids. Respiratory mucus also contains a host of antimicrobial factors (2). The mucus layer is most pronounced in the intermediate to large airways and is approximately 2 to 10 μm thick in the trachea (2). The major constituents of mucus are gigantic peptidoglycan biopolymers known as mucins. These mucins provide the structural framework of the mucus barrier and are largely responsible for the rheologic properties of mucus. They prevent barrier dehydration, sequester pathogens, and act as a reservoir for host-protective proteins and peptides (1). With molecular weights ranging from 3 to 7 million Da, these enormous hydrophilic molecules consist of a core polypeptide chain plus numerous sugar side chains. In fact, 70% of their mass is composed of carbohydrates (1). Mucin macromolecules are well suited for trapping inhaled particles, at least in part, due to diversity of their carbohydrate side chains, which essentially provide a library of ligands for pathogen binding (3). Intramolecular and intermolecular bonds, including disulfide linkages, and ionic and sugar-sugar

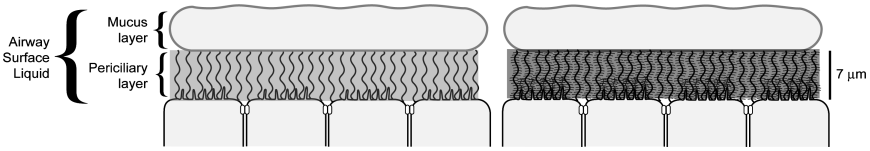

Figure 1 Traditional and revised model of the ASL. The ASL is composed of the mucus layer and the PCL. The mucus layer is viscoelastic, heterogeneous gel layer primarily composed of gel-forming mucins that serve to trap inhaled particles for clearance. Traditionally, the PCL was thought to be an aqueous solution that facilitated cilia beating (*left image*). A more contemporary "two-gel" view depicts the PCL more accurately as a grafted-gel structure of cell surface (tethered) mucins and glycolipids that coat the cilia and microvilli (*right image*). The gel-like PCL not only serves as a lubricating layer for cilia, but also as a barrier between inhaled particles and the cell surface through the protection provided by close approximation of the long-branched mucins. *Abbreviations*: ASL, airway surface liquid; PCL, periciliary layer.

interactions produce a complex, tangled structure that translates into tangible viscoelastic properties (4).

MUC5B and MUC5AC are the major gel-forming, secreted mucins produced in the lung and together comprise approximately 90% of the mucin content of sputum (5), both in pathologic and normal states. Only small amounts of MUC2, another secreted mucin, have been detected in the lung (5). MUC5B is typically thought to originate from submucosal glands, whereas MUC5AC is produced and released by goblet cells of the surface epithelium. These cell types are normally restricted to large, conducting airways. The concentration of mucus-secreting cells in the larger airways brings into question the source and necessity of mucus for the defense of small airways, and the possibility that other cell types (e.g., Clara cells) could be an additional source of mucus in these critical lung regions.

Mucin secretion from submucosal glands and superficial epithelia appears to be prompted by distinct stimuli (6,7), suggesting that the mucin composition of mucus may be influenced by the relative contribution from each source (1). It has been speculated that MUC5AC may be an acute-response mucin that is produced in response to insults to the upper and central airways (1). In fact, MUC5AC mRNA and protein expression are upregulated by neutrophil elastase, a protease secreted by neutrophils during inflammation (8). MUC5B, on the other hand, has been postulated to play a larger role in the setting of chronic inflammation and persistent infection (1). Confirming these speculations, both MUC5AC and MUC5B are found in the mucus plugs that obstruct CF airways, and both molecules are variably increased in induced sputum from patients with CF and COPD. Further, a low-charge glycoform of MUC5B is also elevated in these disease states and might impact the physical properties of the mucus gel (9,10).

After synthesis, MUC5B and MUC5AC are stored in intracellular membrane-bound granules and await secretion via either constitutive or stimulated mechanisms. This allows the epithelium to respond rapidly to environmental challenges, with no requirement for de novo mucin production (1). Secretion of glycoproteins from submucosal glands is predominately under cholinergic control, although adrenergic agonists and multiple inflammatory mediators can also contribute to release. Mucin secretion

from goblet cells, on the other hand, is triggered by increased intracellular calcium levels and therefore responds to stimulation of luminal $P2Y_2$ nucleotide receptors. Certain physiologic conditions can also increase mucus gland secretion, including (*i*) hypoxia, (*ii*) stimulation of mechanoreceptors in the stomach, (*iii*) stimulation of cough receptors in the trachea and bronchi with chemical agents, and (*iv*) inhalation of a wide variety of irritants (cigarette smoke, ammonia, etc). Many of the stimuli that increase mucus secretion also elicit cough, suggesting that the two mechanisms are linked.

Under normal conditions, mucus efficiently protects the airways. However, there are situations in which mucin secretory cell hyperplasia occurs as an adaptive response to chronic inflammation. In chronic airway diseases such as CF, considerable hyperplasia of mucus secreting elements can lead to a pathologic increase in mucus production. In the extreme state, such as status asthmaticus, massive mucin hypersecretion coupled with airway smooth muscle contraction can lead to complete airway obstruction and death (11,12).

Although the importance of mucins should not be underestimated, there is more to the airway mucus than mucins alone. This is evidenced by the fact that concentrated solutions of mucins, in isolation, do not reproduce all the physical properties of the mucus gel (13). Instead, mucus is a complex mixture of ions, mucins, glycoproteins, proteins, and lipids. In disease states, this biologic mixture becomes even more complex. It is estimated that there are more than 100 nonmucin proteins present in the mucus layer, with larger numbers expected in the setting of airways disease (14). Although a full characterization of the nonmucin components in the mucus layer has been slow to emerge, it is likely that the number of globular proteins and their associated functions have been greatly underappreciated and are worthy of further investigation.

Another critical constituent of mucus lining airways is water. Normal mucus is approximately 98% water (2% solids), and even relatively small changes in water content (e.g., from 98% to 94%) can dramatically alter the physical properties of a mucus gel. After secretion, tightly packaged mucin molecules rapidly expand and form a hydrated gel. The characteristics of this gel are in part determined by the ionic composition and pH of the milieu it encounters upon secretion. Aberrant mucin hydration is of particular interest in patients with CF. Specifically, the water imbalance in epithelial fluid may negatively impact mucin unpackaging and hydration, which may help explain the abnormal properties of CF mucus (15).

III. The Periciliary Layer

Traditionally, the PCL has been conceptualized as a simple aqueous solution that provides a low viscosity environment conducive to ciliary beating (16). More recently, the PCL has been envisioned as a highly ordered polyelectrolyte gel. The molecules comprising this gel in the periciliary space include tethered mucins, particularly MUC1, MUC4, and MUC16 (17), which possess transmembrane domains that fix them to epithelial cell membranes that cover microvilli and cilia. These tethered mucins are polyanionic, and repulsive forces cause them to take an extended, brush-like configuration around the cilia (Fig. 2). This PCL model is extremely attractive for several reasons. First, the polyelectrolyte brush configuration is expected to dramatically lower frictional forces between beating cilia (16,18) while also providing a means to mechanically couple their movements. Second, a gel-like PCL provides a mechanism

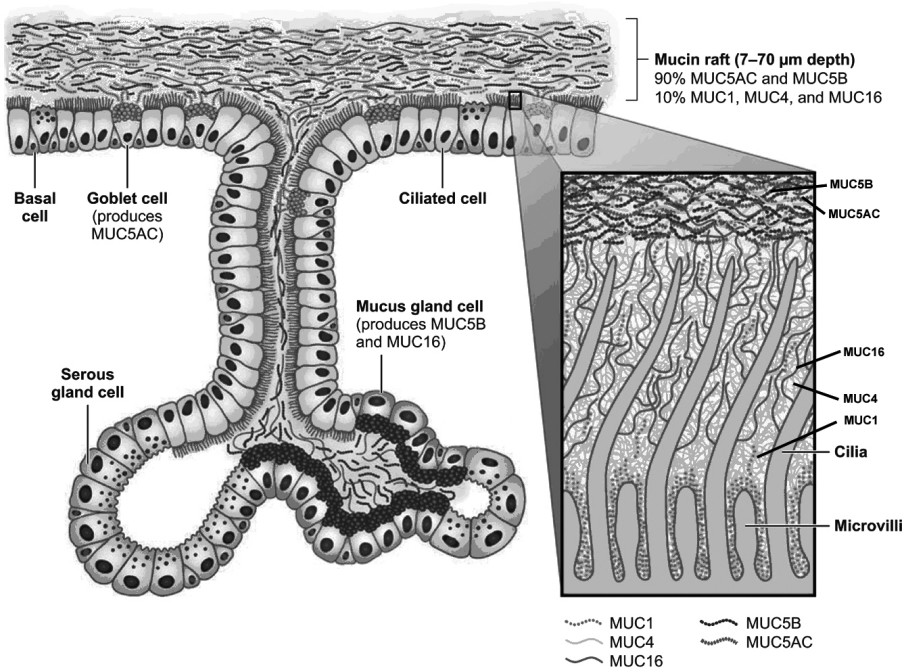

Figure 2 Mucins on the respiratory epithelium. Tethered mucins (MUC1, MUC4, and MUC16) are found primarily along the cell surface in the PCL, but also comprise about 10% of the overlying mucin raft (layer). MUC5AC (from goblet cells) and MUC5B (from submucosal mucus glands) account for the large majority of the mucus raft. *Source*: From Ref. 17.

that prevents penetration of the mucus layer into the PCL environment and subsequent adhesion to cell surfaces. Third, the PCL gel serves a barrier function against inhaled pathogens with particles larger in diameter than the space between tethered mucin molecules being excluded (i.e., >20–30 nm). Finally, this configuration better explains the observation that excess fluid on airway surfaces does not cause separation of the PCL and mucus layers (i.e., mucus floating off cilia tips) and the consequent predicted decline in cilia-driven clearance (19). Rather, the mucus and PCL layers remain in proximity to each other and mucus transport is increased. The same phenomenon is observed in vivo in the case of patients with pseudohypoaldosteronism (PHA), in whom inherited defects in the epithelium sodium channel (ENaC) cause increased amounts of airway lining fluid and dramatically accelerated rates of mucociliary clearance (MCC) (20). Therefore, current data suggests that once the PCL is fully hydrated, additional water is absorbed by the sponge-like mucus layer, making it more easily transportable. In contrast, when moderate airway dehydration occurs, the mucus layer is able to donate water to the PCL to support its functions. This two-gel system is likely stable until extreme dehydration occurs and the osmotic pressure of the mucus layer exceeds that of

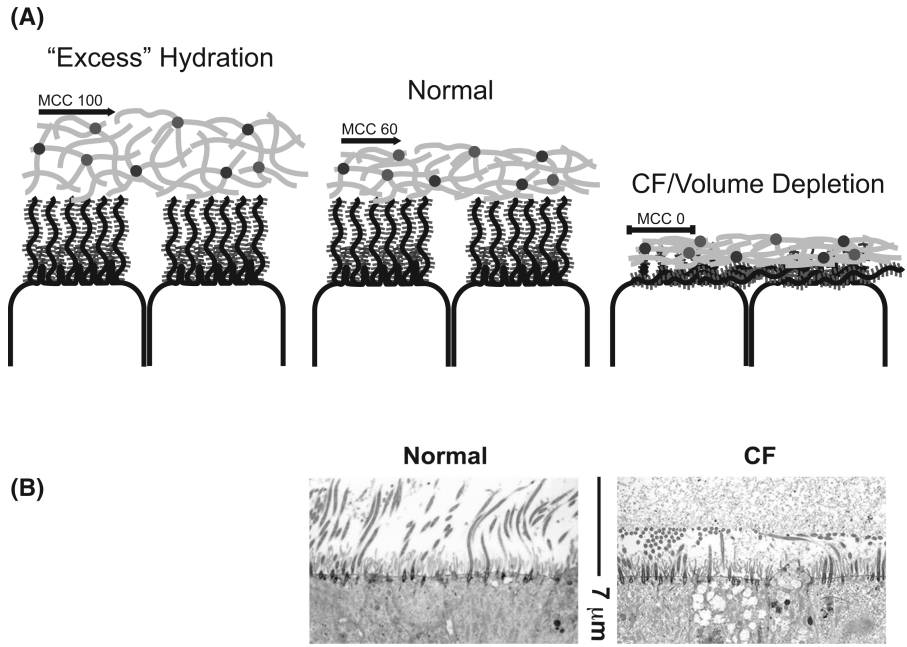

Figure 3 (**A**) ASL response to different states of hydration. When there is excess fluid on the airway surface, the mucus layer absorbs it which makes it more transportable. With dehydration, the mucus layer is concentrated and encroaches into the PCL. (**B**) Electron micrograph of normal versus cystic fibrosis respiratory epithelia. The normal PCL is adequately hydrated, allowing full extension of cilia. The CF PCL is dehydrated and collapsed, altering cilia configuration and impairing motility. A concentrated mucus layer is seen above the PCL in the CF micrograph. *Abbreviations*: ASL, airway surface liquid; MCC, mucociliary clearance; CF, cystic fibrosis; PCL, periciliary layer.

the PCL, causing subsequent collapse of the PCL and encroachment of mucin into this space (Fig. 3).

IV. Mucociliary Clearance

Inhaled particulates and pathogens routinely reach the lower airways, but are typically trapped within the mucus layer and transported out of the lung via mucus clearance mechanisms without the need to mount a potentially deleterious inflammatory response. Mucus clearance, therefore, is widely considered to be the primary innate airway defense mechanism (3). Perhaps the clearest example of the direct relationship between defective MCC and disease is provided by patients with primary ciliary dyskinesia (PCD), who have defective or absent ciliary activity. Over time, these patients develop chronic lower airway infections and bronchiectasis, as well as significant sinus and middle ear infections. Other examples of diseases in which defective mucus transport is felt to play a role in pathogenesis are chronic bronchitis, ventilator-associated pneumonia, and CF. Clearly, the failure to clear mucus renders the airways vulnerable to

infection, as the antimicrobial substances present in mucus alone are incapable of chronically suppressing bacterial growth in the lung (3).

Mucus is transported over airway surfaces via cilia and airflow-driven mechanisms. Cilia-mediated mucus clearance requires an intact PCL and is related to both ciliary beat frequency and mucus rheologic properties needed for optimal mucus transportability. An abnormality in either ciliary beating or mucus rheology can result in defective mucus transport. Cough clearance is independent of ciliary function, but instead requires the generation of adequate airflow velocity as the propulsive force. Cough, therefore, is most effective in the central airways, where airflow velocity is greatest. Like cilia-mediated clearance, cough clearance also is dependent on ASL properties. Cough clearance increases as the hydration of the ASL increases, but decreases with increased ASL viscosity (3). The preservation of cough clearance in PCD and COPD may explain why these diseases are less severe than CF, where both cilia and cough-mediated clearance are impacted by severe ASL dehydration.

Given the importance of mucus clearance, it is not surprising that airway epithelial cells coordinate the relevant effector mechanisms (3). The basal rate of MCC is a function of cilia beat frequency and the properties of the overlying mucus, and there are autocrine and/or paracrine airway epithelial signals that help regulate these properties. For example, adenine nucleotides are released by airway epithelia in response to mechanical shear stresses created by tidal respiration and cough (3,21,22). After release, adenosine 5′-triphosphate (ATP) binds to $P2Y_2$ receptors and, via calcium signaling pathways, increases cilia beat frequency and chloride/water secretion. The end result of these physiologic processes is accelerated mucus clearance. Metabolism of ATP by extracellular nucleotidases produces adenosine, which in turn stimulates chloride secretion via CFTR after binding to the A_{2B} adenosine receptor and increasing intracellular cAMP. Likely, other extracellular signals influence MCC as well, perhaps working through distinct signaling pathways.

Mucociliary clearance measurements have been performed for years using various methodologies. The inhalation of aerosolized, radiolabeled particles followed by imaging with γ-scintigraphy is the most widely accepted method, although until recently there has been little standardization of the technique. This has limited the comparison of results published by different research groups (23). Even so, multiple studies have clearly demonstrated that patients with CF and COPD have defective mucociliary clearance (24). Interestingly, in COPD, the observed mucus clearance defect is most notable in study parameters that reflect large airway clearance (25). However, these patients appear to have intact small airway clearance and robust cough-induced clearance. In contrast, studies of patients with CF have revealed mucus clearance defects that are more pronounced in the small airways, along with markedly reduced cough clearance (26,27). These data, therefore, provide insights into the distinct pathophysiology of these diseases and suggest targets of tailored therapeutic interventions.

V. The Airway Surface Liquid in Cystic Fibrosis

The abnormal properties of CF secretions are striking and give rise to duct obstruction in multiple organ systems. However, identifying the mechanisms that underlie this phenomenon in the lung has been problematic because separation of the primary pathophysiologic processes and secondary effects of infection and inflammation is often

difficult. In fact, most of the previously cited qualitative mucin abnormalities (elevated fucose content, decreased sialic acid content, increased sulfation) appear to reflect the presence of chronic infection and inflammation, rather than a CF-specific defect (28). The current prevailing hypothesis that links CFTR dysfunction to the development of lung disease proposes that dehydration of the PCL and mucus layer, because of altered ion transport, leads to mucus stasis in the lung and subsequent infection and inflammation. Ion transport abnormalities in CF have been thoroughly documented and include the absence of cAMP-mediated chloride secretion (via CFTR) and excessive sodium absorption through EnaC (22). The predicted net effect of altered CFTR and ENaC activity is reduced ASL volume. Support for this hypothesis comes in part from in vitro studies that demonstrate reduced PCL height in cultured CF airway epithelia using confocal microscopy (29). Additional support comes from the creation of transgenic mice that overexpress an ENaC subunit and has accelerated sodium absorption from across airway surfaces. These animals have a reduced ASL height, dehydration of airway secretions, slowed mucus transport, and mucus adhesion with plugging of airways. Interestingly, these mice also develop neutrophilic airways inflammation without overt bacterial infection, suggesting a potential direct link between mucus retention and the development of airway inflammation (30). Finally, data from patients with CF show that these patients do indeed have dehydrated airway secretions and abnormal mucus transport (27,31,32).

The more refined view of the ASL as a "two-gel" system allows us to better understand the effect that ASL hydration has on airways function. Using in vitro airway models, studies have revealed that the volume depletion associated with CF occurs sequentially, first from the mucus layer and then from the PCL (19). With progressive dehydration, the osmotic pressure of the mucus layer eventually exceeds that of the PCL, forcing water movement from the PCL into the mucus layer, causing a reduction in PCL height/volume. Similarly, rehydration of airway surfaces, after ASL dehydration has occurred, first corrects the deficiency in PCL volume. Addition of volume thereafter preferentially swells the overlying mucus layer.

The consequences of PCL dehydration in CF are likely twofold. First, and perhaps most importantly, once the PCL becomes volume depleted, the overlying mucus layer encroaches into the near-cell environment, and the secreted mucins, MUC5AC and MUC5B, begin to interact with the cell-attached mucins. Interaction between secreted and tethered mucins, and perhaps other cell surface molecules (e.g., glycocalyx), causes adhesion between the mucus layer and cell surfaces, resulting in the development of mucus plaques and plugs. Second, PCL volume contraction distorts the space in which cilia beat, thereby reducing or eliminating the propulsive force normally provided by beating cilia. Importantly, these phenomena are expected to interfere with both cilia and cough driven clearance. Exacerbating the problem, goblet cells and glands continue to secrete mucus in diseased airways, worsening the buildup of mucus. Static endobronchial mucus provides a fertile nidus for infection and, ultimately, motile *Pseudomonas aeruginosa* penetrates into mucus plaques and thrives in the privileged environment that they create. *Pseudomonas* quickly adapts to this unique environment, which includes regions characterized by significant hypoxia, by producing an alginate coating, significantly altering gene expression, and thereafter persisting as a bacterial biofilm. The ultimate result is a chronic, incurable bacterial infection.

The effect of ASL dehydration on the mucus layer should also be considered. Once again, in vitro studies demonstrate that once airway secretions increase beyond approximately 6% solids (normal secretions are approximately 2% solids, 98% water), they become much less transportable. This degree of dehydration is in fact quite relevant, as airway secretions from stable adults with CF are often in the 5% to 10% solids range, with even higher levels observed in secretions harvested directly from airways at the time of lung transplantation. Dehydration of airway mucus also results in a reduction in the pore size of the mucin mesh network. When comparing normally hydrated and dehydrated airway mucus (i.e., 2.5% vs. 6.5% solids), neutrophil migration essentially ceased at the higher mucus concentrations, and bacterial capture and killing were significantly impaired (33). Concentrated airway mucus (8% solids) has also been shown to promote the development of *Pseudomonas* biofilms by limiting bacterial motility and the diffusion of small molecules, and to further the impairment of secondary immune defenses (e.g., lactoferrin) in the lung (34). These findings may also help to understand the accentuated inflammatory response that has been observed. If mucus is too concentrated to allow penetration and bacterial killing by neutrophils, the cells can remain in an activated state and continue to release cellular products (e.g., elastase) that ultimately damage airways and stimulate mucus secretion. Finally, as neutrophils degrade, they release DNA into the extracellular environment, which is a further impediment to the clearance of secretions because of its effects on mucus viscoelasticity.

Although the number of mucus-secreting cells increases in CF, it is worth noting that there does not appear to be a major change in the cellular distribution of MUC5AC and MUC5B. It should also be mentioned that there are significant technical difficulties associated with mucin immunodetection. Not only are these molecules very large and highly glycosylated, but the generation of robust antibodies also has been difficult, and the CF airways environment itself is replete with proteases that degrade mucus proteins and reduce our ability to detect them using the antibodies that are available. As a result, some investigators have reported that CF is associated with a reduced quantity of mucins, leading them to the hypothesis that this could represent a host defense defect in and of itself (35,36). In contrast, others have shown that an abundance of mucins are present in CF secretions (9,10). Further experiments done in the presence of protease inhibitors and using methodologies that do not rely on antibody detection (i.e., mass spectroscopy) may shed light on this issue.

VI. Cilia in Cystic Fibrosis

In one of the first examinations of the ultrastructural features of respiratory cilia in CF, cilia from patients with CF were noted to appropriately contain dynein arms and radial spokes (37). Minimal ciliary abnormalities were detected including compound cilia, cilia with excess cytoplasmic matrix, rippled cilia, and cilia with abnormal number or arrangement of microtubular doublets (37). Except for the slightly higher occurrence of rippled cilia in these patients, the abnormalities were similar in morphology and incidence to that of a control group of patients with chronic bronchitis (37). Unmistakably, patients with CF do not have ultrastructural ciliary defects like those seen in PCD.

Nasal ciliary function and mucociliary clearance have been studied in patient cohorts with CF, sinusitis, non-CF bronchiectasis, and aged-matched controls. Ciliary beat frequency was slower in the patients with non-CF bronchiectasis ($p < 0.05$)

compared with patients with CF, sinusitis, and aged-matched controls (38). Nasal mucociliary clearance in CF and non-CF bronchiectasis was slower than that of controls ($p < 0.001$) and patients with sinusitis ($p < 0.01$) (38). The finding of a normal beat frequency in CF cilia studied in vitro in combination with abnormal nasal mucociliary clearance measured in vivo in the same patients suggests an abnormality of mucus, rather than of the cilia themselves.

More recently, nasal mucociliary clearance in children with CF was studied and compared with that of children with PCD and with simple cardiac but no respiratory disease (considered negative controls). No difference was seen in nasal MCC times between children with CF and those without respiratory disease (39). However, those with PCD universally had delayed nasal MCC times. An adult CF cohort was also examined after being divided into those with and without chronic sinusitis (39). Those with sinusitis, which included 43% of CF adults, had longer nasal MCC times than those without chronic sinusitis (39). These data suggest that there does not appear to be a primary impairment in cilia-mediated mucus clearance in CF subjects. Instead, there is likely secondary impairment of cilia-mediated mucus clearance as a consequence of longstanding mucosal inflammation as evidenced by longer nasal MCC times in adults compared with children with CF, particularly in adults with chronic sinusitis.

Therefore, virtually all studies agree that ciliary morphology and function in CF airway epithelia are intrinsically normal (37). This is in stark contrast to PCD in which congenital defects in ciliary structure and function lead to impaired mucociliary clearance and repeated respiratory tract infections (40). The lack of cilia-mediated mucus clearance in PCD leads to heavy dependence on cough clearance alone. Patients with CF, in contrast, have impaired mucociliary clearance due to volume depletion of airway surfaces. With progressive dehydration, the PCL fails to support transport of the airway mucus layer, resulting in mucus adhesion. This is catastrophic, because not only are cilia unable to function in this configuration, but cough clearance fails as well.

VII. Therapies for Defective Mucociliary Clearance in CF

A number of novel CF therapeutics are aimed at improving ASL hydration by either stimulating increased epithelial liquid secretion (e.g., hypertonic saline, dry powder mannitol, denufosol tetrasodium, and small molecules that restore mutant CFTR function), or slowing ASL absorption (e.g., amiloride, PS-552, and other ENaC inhibitors). Together, these novel therapeutics represent a substantial portion of the CF drug discovery pipeline, and signify that an improved understanding of disease pathogenesis is now being translated into interventions that target the underlying cause of disease.

In the case of hyperosmotic hydrators, inhaled hypertonic saline and dry powder mannitol have both been shown to improve mucociliary clearance in proportion to the administered dose (41,42). The basis of this dose-response relationship likely reflects a direct relationship between the number of osmoles deposited on airway surfaces and the resulting magnitude and duration of the ASL volume response. Unfortunately, the tolerability of inhaled hyperosmotic solutions is also related (inversely) to the administered dose, with pharyngeal irritation, cough, and bronchospasm being the usual limiting symptoms. Importantly, hypertonic saline has been shown to improve lung function and significantly reduce disease exacerbations (43). Currently, studies are being performed

that will test the impact of hypertonic saline in CF infants, where the greatest potential benefit may be realized.

Sodium channel blockers (amiloride, PS-552) are antagonists of ENaC and work to slow absorption of sodium from the airway lumen. Although amiloride has not been shown to have clinical efficacy in CF (27,44,45), longer-acting, more potent ENaC blockers are now being tested (46). It is also intriguing to consider whether an ENaC inhibitor could act synergistically with a drug that stimulates ASL secretion by accentuating the size and/or duration of the resulting ASL volume response.

Denufosol tetrasodium, a selective $P2Y_2$ receptor agonist, bypasses the defective CFTR chloride channel by activating an alternative chloride channel (calcium-dependent chloride channels or CaCC). This is predicted to result in an increase in airway surface epithelial hydration. It appears to be therapeutically promising (47) and is currently in phase 3 clinical development. How it might interact with hyperosmotic agents is unknown and difficult to predict a priori, but will be an important issue if approved for clinical use.

Recombinant human deoxyribonuclease I (rhDNase, or Pulmozyme), a cloned enzyme that cleaves the DNA residue left by degenerating neutrophils thus reducing sputum viscosity, is another means of altering mucus properties in CF (48). The enzyme was approved by the U.S. Food and Drug Administration in 1994 for use in CF patients. It has since accumulated a considerable history of safe administration and robust evidence for efficacy in CF patients across the disease spectrum. Other therapeutics that target mucus adhesion or other adverse rheologic properties in CF constitute another exciting prospect that is being explored.

Perhaps most exciting is the possibility of restoring CFTR function, the most basic defect in CF pathophysiology. An enormous amount of work has gone into identifying orally available small molecules that have the capacity to correct mutant CFTR processing (i.e., ΔF508) and CFTR function (class III and IV mutations), or to promote readthrough of CFTR stop mutations. These new therapeutic classes, designed to address specific CFTR mutations and systemically restore CFTR function rather than ameliorating the sequelae CFTR dysfunction, hold great promise for revolutionizing the care of CF patients in the future. They are another prime example of how improved understanding of disease pathogenesis is driving the development of therapeutics in CF, particularly at the level of early disease pathogenesis.

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