CF and lung homeostasis

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CYSTIC FIBROSIS (CF) is a devastating disease of the lungs, intestines, pancreas, and other organs that is characterized by viscous secretions of the exocrine glands (reviewed in Ref. 18). The gene responsible was cloned in 1989 and named the cystic fibrosis transmembrane conductance regulator or CFTR (64). In CF, a number of organ systems are affected; however, the most severe pathological consequences are lung associated. CF patients present with dehydrated mucus in the lungs that leads to airway obstruction, chronic bacterial infections and inflammatory responses, bronchiectasis, and eventually respiratory failure (21, 65). CFTR is a cAMP-activated chloride (2) and bicarbonate channel (59). Although it is clear there is a chloride/bicarbonate defect in CF, there is significant support for the idea that CF lungs have elevated amiloride-sensitive sodium reabsorption and that CFTR regulates the epithelial sodium channel (ENaC) activity (7, 8, 37, 41, 43, 44, 52), although the ENaC effect in CF is controversial (14, 38) (reviewed in Ref. 18).

ENaC and CFTR

Recent studies have examined the role of ENaC dysfunction in CF in a number of different ways. A study by Rubenstein, Grumbach, and coworkers (66) examined the regulation of endogenous ENaC by wild-type (WT) and ΔF508 CFTR. ΔF508 CFTR is the most common mutation found in CF patients and results in a misfolded protein that is degraded by the endoplasmic reticulum (ER)-associated degradative pathway and the proteasome (76). In this study, the authors examined why the ENaC functional activity was elevated in CF airway epithelia. Their CF airway epithelial model system was the CFBE41o− parental cells that are homozygous for the ΔF508 CFTR mutation (9) and ΔF508-CFTR overexpressing cells. These were compared with CFBE41o− WT-CFTR overexpressing cells. All of these CFBE41o− cells have little ENaC expression, so ENaC expression was induced with a 24-h treatment with 1 μM dexamethasone (Dex). Functional ENaC as monitored in Ussing chambers was absent in parental CFBE41o− and CFBE41o− ΔF508-overexpressing cells but present in all Dex-treated cells. Two significant points were made here. First, Dex treatment did not activate the ENaC channels in CFBE41o− WT cells, indicating that overexpressed WT CFTR effectively suppresses ENaC activity. Second, Dex-treated CFBE41o− cells with ΔF508 CFTR (parental and overexpressed ΔF508 CFTR) have elevated ENaC activity (66).

In another approach using mouse knockout models, Lazrak and coworkers (46) compared WT (Cftr+/+) heterozygous (Cftr+/−), knockout (Cftr−/−), and ΔF508-expressing mice (CftrΔF508) for ENaC activity. Using freshly prepared lung slices from the animals, they monitored single-channel activities of ENaC using whole cell patch clamp on alveolar type II (ATII) cells and demonstrated that the ENaC basal open probabilities were vastly different between the different mouse groups. The basal open probabilities were WT = 0.21, Cftr+/− = 0.4, CftrΔF508 = 0.55, and Cftr−/− = 0.81. They also monitored alveolar ENaC activation by forskolin and trypsin in the lung slices and found that the relative amount of ENaC activity was inversely correlated with the predicted CFTR levels in these mice. Interestingly, there were significant differences between the WT and heterozygous mice, as well as between the knockout mice and the ΔF508 CFTR mice, suggesting an inverse relationship between CFTR and ENaC activities. Western blot analysis revealed that the amount of lung α-ENaC in each of the animal groups was similar, suggesting that the level of activation of the ENaC channels or their surface expression levels were different in each of the animal groups (46). Another interesting result from this study is that Lazrak et al. (46) were not able to detect single channels consistent with CFTR activity in the cell-attached mode (46), suggesting that the amount of CFTR is very limited in these cells and therefore extremely small amounts of CFTR activity have profound suppressive effects on ENaC activity. This is consistent with earlier reports that CFTR expression is extremely low in ATII cells in rodents (15, 78).

Can dysregulation of ENaC result in a CF-like lung disease? The answer comes from the development of the β-ENaC mouse model (53). Mall, Boucher, and coworkers (53) over-

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expressed the β-subunit of ENaC in club (Clara) cells to target the lower airways. These animals had elevated ENaC activities, normal cAMP-activated and calcium-activated chloride activities, but, nonetheless, many of the classic features of CF lung disease including reduced periciliary liquid height, reduced mucus transport and bacterial clearance, airway obstruction, and a high postnatal mortality rate. As a further test of this model, Grubb, Boucher, and coworkers (31) tested the idea that transgenic human CFTR expression could correct the β-ENaC mouse lung disease. Interestingly, in spite of the fact that the transgenics were made with the club cell secretory promoter (the same promoter used in the β-ENaC mice), and the basal chloride channel activity was elevated 2.5-fold, human CFTR gene expression failed to rescue the lung phenotype (31). Why human CFTR in this case this failed to rescue the overactive ENaC activity and phenotype, however, is not entirely clear.

In the original β-ENaC mouse with overexpressed ENaC, the endogenous mouse Cftr gene is still present, yet there is a strong lung phenotype, indicating that mouse CFTR cannot rescue overexpressed ENaC. Even inserting human CFTR in the same cell type does not rescue ENaC overexpression. Furthermore, Cftr knockout mice do not exhibit the human CF lung phenotype, a problem that has been associated with the CF mouse model. The lack of a CF phenotype perhaps is why the β-ENaC mouse is so appealing. Given that, Grubb and colleagues (50) tried a different approach. In this case, they asked whether the double-mutant ΔF508 CFTR/β-ENaC mice would have a more severe lung phenotype than the β-ENaC mouse. Survival of the double-mutant mice was reduced compared with survival of either single mutation. The double-mutant mice also exhibited higher neutrophilic pulmonary inflammation, suggesting that the increased sodium absorption and decreased chloride secretion are additive effects in the development of the lung pathology (50).

Can mutations in ENaC generate CF symptoms in patients? Interestingly, Bours and colleagues (56) identified a patient with CF-like symptoms that had a mutation, V348M, in the β-subunit of ENaC. To examine the mechanism behind this defect, Korbmacher and colleagues (62) expressed WT αβγENaC or ENaC with this mutation, αβV348MγENaC, in Xenopus laevis oocytes. Analysis of this mutation revealed that the mutation increased the open probability of the sodium channel. The gain-of-function mutation was confirmed in HEK293 cells, and the results provide support for the idea that patients with atypical CF could have a combination CF allele and a gain-of-function mutation in ENaC (62). All of this is consistent with early reports by Knowles, Boucher, and colleagues (8, 41) that demonstrated the importance of sodium reabsorption in cystic fibrosis respiratory epithelia.

**Mucus and Fluid Transport**

A central element in the lung pathology in CF is compromised mucociliary clearance. This results in a breakdown in the innate immune response to remove pathogens from the surface of lung and leads to a chronic inflammatory response (reviewed in Ref. 49). The airway surface is covered with a thin layer of liquid and mucus called the airway surface liquid (ASL). Ciliated cells pulse continuously to move mucus and associated bacteria toward the pharynx to cleanse the airways. Below the mucus layer is periciliary layer (PCL) that needs to be properly hydrated for effective cilia function (49). This requires that the PCL be the same height as the cilia (~7 μM) (49). A delicate balance between CFTR chloride/bicarbonate secretion and ENaC sodium absorption is believed to maintain this proper PCL height and alterations in either one can dehydrate the airway surface and interfere with mucus clearance (49). There is controversy, however, about the decreased ASL height in CF and the mechanisms involved in generating the viscous mucus (14, 22). For example, previous studies by Zabner, Welsh, and colleagues (14) indicated that, in the CF pig model, sodium and water absorption as well as ASL height were not affected in CF pigs. Furthermore, studies by Verkman and colleagues (22) support the view that the ASL height is not affected in CF, whereas the viscosity is.

More recently, Zabner, Welsh, and colleagues (47) examined CFTR’s role in transepithelial liquid transport in the CF pig. Using isolated type II alveolar epithelial cells and ex vivo studies of newborn lungs, their data indicated that CFTR was not required for basal liquid absorption but was required for cAMP-stimulated liquid absorption and secretion, suggesting that the apical liquid layer is controlled by a number of ion channels at both the apical and basolateral membranes (47). These results are consistent with previous results of Matthey and coworkers (19, 25), who suggested that CFTR is important for apical-basolateral fluid transport in cultured human alveolar epithelial type II cells.

A major problem in CF is reoccurring bacterial infections and the most common pathogen is *Pseudomonas aeruginosa* (21). In response to this infection, two cytokines are released, interleukin-1β and tumor necrosis factor-α. Machen, Ianowski, and colleagues (3) examined the connection between these cytokines and CFTR’s role in fluid secretion. Their rationale was that these cytokines are released during *P. aeruginosa* infections that are common in CF Airways. Their results suggest that both cytokines independently stimulate CFTR-mediated fluid secretion from swine airway submucosal glands. The significance of the studies is that this normal fluid secretion in response to bacterial infection would not be operational in CF patients.

CF patients constantly deal with bacterial lung infections and are often treated with macrolide antibiotics for *Streptococcus pneumoniae* and *Haemophilus influenzae* (39). One interesting feature of these macrolide antibiotics is that they also have immunomodulatory and anti-inflammatory properties. Tarran, Wilbert, and colleagues (72) took an interesting approach and dealt with the issue that chronic antibacterial use often promotes bacterial resistance. To circumvent this problem, they designed a novel antibacterial macrolide, 2’-desoxy-9-((S)-erythromycylamine (GS-459755), and tested its effects on inhibiting human neutrophil elastase (HNE)-induced mucus stasis that is caused by activating ENaC. Their results indicated that GS-459755 pretreatment blocked HNE-induced ASL volume depletion in human bronchial epithelial cells. This suggested that this type of drug treatment could be useful in the treatment of mucus dehydration in both CF and chronic obstructive pulmonary disease (COPD) without the confounding effects of bacterial resistance induction (72).

One proposed model in CF suggests that the increased viscosity of gland secretions is caused by the reduced fluid secretion by serous cells in a background of normal mucus secretion by mucous cells (28). To test this hypothesis, Fink-
beiner, Widdicombe, and coworkers (28) looked at the relationship between mucin and chloride secretion by human airway gland mucous cells. Their previous studies had shown that mucous cells secrete as much chloride as serous cells in culture (27). They also found that mucous cell secretions that were induced after activation of β-adrenergic receptors are more abnormally concentrated in CF cultures compared with normal airway mucus cells, whereas mucin stimulation by other pathways, e.g., purinergic, cholinergic, or α-adrenergic, were unaffected in CF (28). This indicated that mucin secretion is independent of chloride secretion in mucous cells and that the ratio of chloride to mucin secretion varies depending on the mediator (28). This also suggests its altered responses to β-adrenergic responses can account, at least in part, for the viscous mucins in CF (28).

Secretion of mucus in the airway epithelium is mediated by the myristoylated alanine-rich C kinase substrate (MARCKS) (48). A synthetic amino-terminal peptide from the MARCKS called MANS (MARCKS-N-terminal sequence) inhibits mucin secretion in airway goblet cells, indicating that MARCKS is an important component in the secretory apparatus that is required for granule release in a number of cell types (70). A recent study from Davis and colleagues (32), however, questions this model. The MANS peptide is believed to compete with the MARCKS protein for membrane binding and therefore acts as a dominant-negative regulator that blocks mucin release (70). In the Davis studies, the investigators tested this hypothesis by using MARCKS-null mice and harvested cells from mouse embryos since the MARCKS-null mutation results in prenatal lethality (69). Davis and colleagues propose that the peptide interferes with monitoring the secreted mucins rather than blocking mucin secretion as has been proposed (32). This important controversy needs to be resolved in future studies.

Oxidative Stress

Oxidative stress, particularly reiterative oxidative stress such as cigarette smoke, has a number of effects on ion channels (23) and CFTR seems to be particularly susceptible (for review see Ref. 60). Although CFTR expression and function are clearly compromised in CF, it has recently become clear that CFTR is affected by cigarette smoke and that decreases in CFTR expression and function may be a critical component in COPD (12, 16, 24, 42, 68). In a recent study by Vij and colleagues (4), the authors examined the role of CFTR in cigarette smoke-induced lung epithelial injury. Comparing Cfr−/− and Cfr+/+ murine lungs, they found that cigarette smoke induced an increase in ceramide and TUNEL-positive apoptotic cells in Cfr−/− lungs compared with Cfr+/+ lungs, and that defective autophagy from the lack of CFTR leads to apoptosis (4). Given that the CFTR expression losses are induced by cigarette smoke and oxidative damage, they propose that cigarette-induced effects on CFTR are involved in the pathogenesis of emphysema (4).

Inflammation

Chronic lung inflammation is a severe consequence of CF and COPD and is directly correlated with the chronic bacterial infections seen in these two diseases. Clearly, abnormal inflammatory signaling and excessive inflammatory responses are seen in CF patients (5, 54, 55). Kelley, Ziady, and colleagues (79) examined the role of human airway epithelial cells in inflammatory signaling in CF. They had previously reported that the intracellular levels of H₂O₂ were elevated and promoted a hyperinflammatory response in CF epithelia (13). They also reported that a key transcription factor that drives the expression of antioxidant genes, nuclear factor erythroid-derived-like 2 (Nrf2), is reduced in CF epithelia (13). To determine the mechanism responsible for decreased Nrf2 in CF epithelia, they found that cAMP-mediated signaling reduced Nrf2 activity in CF compared with non-CF epithelia (79). Using a cAMP competitor, Rp-cAMP, they demonstrated that Nrf2 activity was rescued in CF cells. They also found that the altered cAMP-mediated signaling in CF decreases the association between Nrf2 and its transcriptional coactivator cAMP responsive element-binding protein (CREB) binding protein (CBP), thereby inhibiting Nrf2’s transcriptional activity. The significance of these studies is the potential for simultaneously blocking the inflammatory responses and shifting the responses to Nrf2 and enhancing antioxidant gene expression (79).

In a different approach, Stanton and colleagues (33) compared the inflammatory responses to Pseudomonas aeruginosa in human airway epithelial cells expressing either WT or ΔF508 CFTR. Unexpectedly, they found that WT CFTR-expressing CFBE41o cells had a greater response to P. aeruginosa infection than ΔF508 CFTR-expressing CFBE41o cells as monitored by IL-8 and TNF-α expression (33). The authors suggest that, given these results, studies on human airway cell lines and primary cells that examine how CFTR mutations affect the inflammatory response to P. aeruginosa infections may not be clinically relevant (33).

CFTR and Inflammation

Bacterial infections and their consequences on inflammatory responses and their consequences in loss of lung function are well known in CF. CFTR is also susceptible to oxidative stress as detailed above. On the basis of recent studies by Londino and colleagues (51), we now also know that influenza suppresses CFTR expression. Given that influenza infections affect a large segment of the US population, the potential for flu epidemics, and the vulnerability of the aging population to flu’s lethal consequences, understanding how flu affects CFTR and lung function is timely and important (reviewed in Ref. 75).

In these studies, Londino et al. (51) demonstrate that the influenza matrix protein M2 decreases CFTR expression and function by increasing the organelle pH in the secretory pathway, leading to ER-associated degradation of CFTR by the ubiquitin-proteasomal system. The M2 protein is a proton channel that acidifies the endosomal compartment to facilitate uncoated of the viral RNA (1). Loss of CFTR channel function would be comparable to oxidative stress effects on CFTR; however, Londino and colleagues showed that the effects of M2 on CFTR expression were not dependent on reactive oxygen species, but on the proton channel activity (51). Interestingly, the M2 protein also inhibits ENaC channels as well by a different mechanism, by increasing reactive oxygen species (45). These studies indicate that influenza affects the activity of two key players in fluid regulation in the lungs and highlight the importance of M2 as a potential therapeutic target in influenza infections (53a).

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Therapies

There are more than 2,000 mutations in the CFTR gene, and most of the previous therapeutic approaches have dealt with limiting the symptoms of the disease rather than directly addressing the cause. Recent therapeutic approaches, however, have focused on gene therapy, ribosomal read-through drugs, correctors, and potentiators (reviewed in Ref. 20). Correctors are small molecules that have been shown to promote the correct folding of CFTR mutations, particularly ΔF508 CFTR, whereas potentiators are designed to increase the open probability of mutations that have a channel gating defect such as G551D CFTR. The most common mutation, ΔF508 CFTR, is found in 90% of the patients, and there is great hope that a combination therapy with a corrector and potentiator may benefit these patients (20). Although much emphasis has been placed on correcting the folding, surface stability, and function of ΔF508 CFTR, it is clear that personalized medicine is the future since all of these other genetic mutations have to be understood and examined as well.

The first step in this process is often testing current correctors for their ability to fix a rare mutation. A recent study by Caldwell et al. (11) examined such a possibility. V232D is a rare folding mutation that can be corrected to near WT, maturely glycosylated protein levels with small molecule corrector Corr-4a (11). Furthermore, single-channel analysis revealed that the chloride conductances were indistinguishable from that of WT, suggesting that this particular mutation is very amenable to correctors currently available that were originally developed for the ΔF508 CFTR folding mutation (11). This type of analysis provides hope that many of the poorly characterized mutations might be amenable to treatment with correctors and potentiators that were developed for the more common folding and gating mutations previously characterized.

A major obstacle with both the correctors and potentiators is determining exactly how they work. Pyle et al. (58) developed an assay for monitoring activation of CFTR based on regulatory domain phosphorylation. This assay is based on the idea that activation of CFTR requires cAMP-mediated activation of protein kinase A (PKA) and subsequent phosphorylation of the regulatory domain (R-D). In this study, they tested nine compounds identified through high-throughput screening to determine whether their mechanism of action required R-D phosphorylation. Their results indicate that there are two types of compounds that can activate CFTR activity, one of which is independent of R-D phosphorylation. This illustrates that augmentation of R-D phosphorylation is not required and that this information can be used to predict which combination of compounds have the potential for synergistic effects (58).

ΔF508 CFTR has a number of defects that have been associated with it, including a failure to exit the endoplasmic reticulum and progress to the cell surface (folding defect), instability at the cell surface if it gets there (folding defect), and a failure of the channel to open properly (gating defect). The folding problems have proven challenging because of two problems in the folding pathway: nucleotide binding domain (NBD) instability and domain interface interactions, both of which are caused by the loss of phenylalanine in NBD1 (30, 35, 57, 61, 63, 74). The complexity of the folding defect has led to the suggestion that more than one corrector or a combination of a corrector and a potentiator will be required to fix the ΔF508 CFTR folding defect (reviewed in Ref. 17). A study by Favia et al. (26) follows this idea by identifying a compound that has both potentiator and corrector activities. Their initial screen of this compound showed that trimethylangelicin (TMA) reduced IL-8 transcription and potentiated CFTR function (71). Follow-up studies showed that nanomolar concentrations of TMA in primary human bronchial epithelial cells from ΔF508 CFTR homozygous patients elevated CFTR-dependent chloride secretion to almost 40% of WT levels, suggesting that single-drug treatments are a possibility (26).

A study by Zhao et al. (77) suggests that other apical channels may be suitable targets for decreasing sodium absorption or increasing chloride secretion. They pose that lack of CFTR in CF leads to hyposecretion of fluid and decreases in ASL height. They also point out that sodium absorption and chloride secretion depend on a favorable electrochemical gradient mediated in part by the basolateral and apical potassium channels, as well as the Na-K-ATPase. In their study, they demonstrate that two-pore K⁺ (K2P) channels are present at the apical membrane of human bronchial epithelial cells and selective inhibitors of these channels could inhibit sodium absorption in the airways, and, conversely, activators of these channels could maximize chloride secretion, either one of which would modulate fluid secretion in the airways.

An alternative approach that has been suggested in CF is to activate an alternative chloride channel. Recent work from Schiffhauer et al. (67) has used such an approach by activating chloride channel protein 2 (CLCN2). Using a CLCN2 transgenic mouse model and Cftr−/− mice, they demonstrate that lubiprostone, an FDA drug approved for idiopathic constipation in adults, can activate CFTR and CLCN2 channels and therefore this drug could be a potential therapeutic if administered as an aerosol (67).

There is a cumulative damage to the lungs in CF over time because of the chronic bacterial infections and continuous inflammatory responses (10). To test whether epithelial repair could be stimulated with an anti-inflammatory mediator, Buchanan et al. (10) tested whether lipoxin A₄ (LXA₄), an eicosanoid formed from arachidonic acid, is beneficial in CF by suppressing the inflammatory damage to the lungs. Lipoxins have been proposed as therapeutics in asthma lung inflammation (6). More importantly, lipoxins have been shown to be significantly lower in airway fluid in patients with cystic fibrosis (40). In the present studies, Buchanan et al. (10) show that LXA₄ increases cell proliferation and migration and wound healing in CF airway cultures and stimulates ATP-activated potassium (KATP) currents to enhance epithelial repair (10). They suggest that this anti-inflammatory response attenuation would be therapeutic in helping to block the lung damage and thereby stabilize lung function (10).

Fixing ENaC is a common theme in CF therapies, and Stutts, Tarran, and colleagues (36) have identified a novel therapeutic to regulate sodium hyperabsorption. A protein called short palate lung and nasal epithelium clone 1 (SPLUNC1) is a secreted protein in airway epithelia that is an autocrine inhibitor of ENaC (36). In this study, Hobbs et al. (36) showed that SPLUNC1 is detectable in CF human bronchial epithelial culture ASL, although it does not appear to be effective in suppressing ENaC activity (73). Tarran and colleagues (29) previously demonstrated that the SPLUNC1 protein’s pH sen-
sitivates interferes with its ability to interact with ENaC. In this study, they show that an 18-residue peptide, S18, from the amino-terminus of SPLUNC1 is sufficient to inhibit ENaC activity and that it binds to the β-subunit of ENaC (36). Importantly, they show that the S18 peptide inhibits ENaC ASL hyperabsorption and that, unlike the intact protein that is ineffective in the CF environment where bicarbonate secretion is compromised, the S18 peptide is pH insensitive (29). Given the effectiveness of the peptide in suppressing ENaC activity, the authors suggest that this is a useful therapeutic for treating ENaC’s effects on the ASL dehydration in CF lungs.

Summary and Conclusions

CFTR’s role in lung homeostasis is dramatically illustrated by its loss in CF. Regulation of other channels as well as regulation of the ASL layer pH and hydration are critical components that need to be corrected in CF. Although there is great promise in current small molecule correctors and potentiators that are becoming available, understanding their mechanism of action and other effects will be an important aspect of future studies. Combination therapies are currently being tested, and clearly this direct effect on CFTR channel folding and function is extremely promising. Whether alternative approaches such as inhibiting ENaC activity will be an additive benefit needs to be examined, especially considering the fact that more than 2,000 separate mutations in the CFTR gene have been described. It is also important to consider CFTR’s importance in COPD since many of the pathological features of these two diseases have common attributes.

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DISCLOSURES

J. F. Collawn has intellectual property rights in an international patent application together with DiscoveryBioMed, on chemical compounds to be used as potential therapeutics in cystic fibrosis. These compounds, however, are unrelated to the chemical compounds discussed here. No other conflicts of interest, financial or otherwise, are declared by S. Matalon.

AUTHOR CONTRIBUTIONS

J.F.C. and S.M. drafted, edited, and approved the final version of the manuscript.

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