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Mucus-targeting therapies of defective mucus clearance for cystic fibrosis: A short review

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Abstract

In the lungs, defective CFTR associated with cystic fibrosis (CF) represents the nidus for abnormal mucus clearance in the airways and consequently a progressive lung disease. Defective CFTR-mediated Cl^- secretion results in altered mucus properties, including concentration, viscoelasticity, and the ratio of the two mucins, MUC5B and MUC5AC. In the past decades, therapies targeting the CF mucus defect, directly or indirectly, have been developed; nevertheless, better treatments to prevent the disease progression are still needed. This review summarizes the existing knowledge on the defective mucus in CF disease and highlights it as a barrier to the development of future inhaled genetic therapies. The use of new mucus-targeting treatments is also discussed, focusing on their potential role to halt the progress of CF lung disease.

1. Mucus transport in normal airways vs. CF airways

Cystic Fibrosis (CF) is characterized by impaired transport of chloride and bicarbonate across the cystic fibrosis transmembrane regulator (CFTR) channel, which results, in the airways, in a critical disease-initiating step: a reduction of mucus transport and clearance [1–3].

In normal airways, an effective mucus transport and clearance requires a well-hydrated airway surface layer (ASL), composed of a mucus layer and a periciliary layer (PCL), which acts as a lubricating layer in which the cilia beat. The water flux from the cells to the ASL is tightly regulated by an interplay of coordinated chloride secretion and sodium absorption across the low-resistance epithelium [4]. Chloride is secreted via CFTR, and the calcium-activated chloride channels (CaCCs) and sodium is absorbed via the epithelial sodium channel (ENaC), while the water is transported passively with the resulting osmotic gradient. Together, the balance of water and ions across the epithelium ensures a properly hydrated ASL and an efficient mucus clearance [5–8].

Mucus production and its clearance are part of our innate immune system designed to protect the pulmonary surfaces from the persistent onslaught of inhaled infectious and noxious substances. Airways mucus can bind to inhaled foreign microorganisms, and the ciliated epithelial cells lining the airways sweep the mucus material until it is either swallowed or coughed out of the airways. This efficient system deters the establishment of lung infections [9–11]. (Figure 1A).

However, in CF airways, defective CFTR function leads to unregulated absorption of sodium and airway fluid resulting in dehydration of both the mucus and PCL layers and abnormal mucus clearance [12]. The dehydration of these layers leads to compressed cilia, impeding their ability to efficiently sweep the mucus and trapped infectious agents out of the airways, thus favoring airway infections. The result is the accumulation of mucus and pathogenic microorganisms, and the development of chronic airway inflammation and progressive lung function deterioration in CF patients [13–15] (Figure 1B).

Importantly, in 2012, our group's research introduced a significant revision to our understanding of the airway surface organization by describing the gel-on-brush model, depicting a high molecular weight secreted mucous layer juxtaposed to airway cell surface-tethered mucins. This work demonstrated that the secreted mucins in the mucus layer, in health, cannot penetrate the PCL as a result of the osmotic forces established by the densely expressed tethered mucins lining the airway surface. However, in CF, the dehydrated airway surface results in an increased mucus osmotic pressure, higher than that of the PCL. The result is water drawn out of the PCL, compression of the cilia, and mucostasis, as mentioned above [5].

In addition, recent studies have indicated that airway mucus obstruction associated with inflammation already occurs during the early stages of CF pulmonary disease. For instance, air trapping has been documented in newborn $CFTR^{-/-}$ piglets devoid of bacterial infection [16], and similar findings were reported in CF infants and pre-school children [17].

2. CF mucus defect

The healthy mucus layer that covers the human airways is a complex mixture of many components, such as mucins, globular proteins, DNA, ions, cells, antimicrobial peptides, salts, and water [18,19]. The interdependency of these components is most apparent in CF airways, where the ion-mediated changes in mucus concentration have a tremendously adverse effect on its overall biophysical properties [20,21]. Here, we explored mucus properties primarily affected by CF: concentration, viscoelasticity, and MUC5B/MUC5AC ratio.

2.1. Mucus concentration

The healthy mucus layer is composed of mostly water (roughly 98%) and only 2% solid materials such as mucins (~0.5%), globular proteins (~0.5%), and salt (~0.9%) [21,22]. In CF, however, as described above, the defective CFTR Cl^- secretion combined with upregulated Na^+ absorption leads to ASL dehydration, which directly results in a higher concentration of mucus solids to values of ~4–5% for expectorated mucus and 12% for

mucus that is collected from freshly excised CF lungs. This higher mucus concentration leads to two main deleterious consequences: 1) increased mucus viscoelasticity and 2) the mucus layer osmotic compression of the PCL. Combined, they reduce mucus clearance which fuels the CF pathogenic loop. Thus, mucus hyper-concentration has a central role in CF disease [2,5,6,13].

2.2. Viscoelasticity

The flowability of mucus is typically described by its rheological properties: viscosity (resistance to flow) and elasticity (stiffness or gel-like properties). In addition, mucus is a non-Newtonian gel that, under high shear stress, behaves as a viscous liquid; thus, the shear properties are essential to maintain its flowable properties [18,19].

As a unique organ, the lungs are constantly subjected to various shear forces during tidal breathing and coughing [23] (PMID: 19166889). For example, during respiration, airflow and transepithelial pressure gradients are two fundamental shear stress forces that act within the surface of airway cells [24,25]. However, more extreme stress can happen on the airways during cough due to mechanical forces, such as transpulmonary pressure. The glottis closure creates this high pressure when combined with the rapid expiratory muscle activity [26].

Changes in mucus rheological properties in the airways can profoundly affect its functioning as a first-line defense against harmful infections. For example, due to the mucus hyper-concentration in CF, the mucus layer's viscoelasticity is higher than normal non-CF values [20]. In addition, the viscoelasticity threshold for which the cilia beating is still effective (100 mPa·s) is surpassed by an airway mucus with concentrations over 2.5% solids [27]. Also, PCL height was shown to decrease when the mucus concentration is around 5% solids, and it completely collapses at concentration above 8% (similar values are found in CF mucus) [5,27]. Therefore, the reduced cilia beat efficiency associated with the osmotic compression of the cilia, momentum transfer from the cilia to the viscoelastic mucus (i.e., motive force) is significantly reduced, resulting in mucostasis and disease progression [27–29].

In addition, the accumulation of static mucus combined with chronic infections in CF airways is associated with inflammation and necrosing neutrophils that release DNA (i.e., Netosis), which further increases mucus viscoelasticity [30,31]. Furthermore, it has also been suggested that the high oxidative stress from neutrophilic inflammation is associated with the formation of additional mucin disulfide bonds, thereby forming a highly cross-linked, and viscoelastic, mucus gel [32].

2.3 MUC5B and MUC5AC mucins in the airways

Mucins are large, multimerized, gel-forming glycoproteins (up to 100 megadaltons in size). MUC5B and MU5AC are the prominent secreted mucin isoforms in human airways produced by submucosal glands (MUC5B) and superficial goblet and club cells (MUC5B and MUC5AC). In health, MUC5B is the dominant mucin isoform which is expressed approximately 75 fold higher than MUC5AC [6]. However, up-regulation of MUC5AC has been demonstrated to be more prominent during pulmonary exacerbations in CF [33], worsening COPD (Gold) status [34], and bacterial [35] and Trichuris muri's (enteric

nematodes) infections [36,37]. Increased MUC5AC has also been associated with allergic inflammation in asthma [38].

It has been suggested that MUC5AC is a “stickier” and stiffer mucin that can be beneficial during pulmonary infections to help bind and clear the inhaled pathogens out of the airways [39,40]. However, numerous data suggest that prolonged and elevated expression of MUC5AC can have detrimental effects on mucus clearance [40,41]. For example, MUC5B knockout mice were shown to have reduced mucus clearance and develop chronic infections that failed to resolve spontaneously [41]. In addition, patients homozygous for a novel splicing variant in the MUC5B gene were found to have impaired mucociliary clearance [42]. Therefore, the role that chronically increased MUC5AC expression plays in disease pathogenesis should be further investigated.

3. Current mucus-targeting treatments in use for CF

3.1. DNAse

As mentioned above, increased DNA released from neutrophils in CF is associated with a higher viscoelasticity. Therefore, an inhaled form of a recombinant human DNAse enzyme that selectively hydrolyzes DNA, named Dornase Alfa, was developed for inhaled delivery. This drug was one of the first approved to treat CF in 1993 and is still commonly used by most patients in their CF management [43,44].

As expected, hydrolyzing DNA with Dornase Alfa is capable at decreasing the viscoelasticity of CF sputum [45]. Clinical studies with Dornase Alfa showed that this treatment significantly decreases pulmonary exacerbations and slightly increases lung function [46]. [44,46]. These positive clinical trial results made Dornase Alfa a standard CF therapy that is widely used today. However, while Dornase Alfa reduces the frequency of pulmonary exacerbations, it does not entirely prevent them from happening. Thus, the development of new mucus-targeting agents continues to be essential to treat CF.

3.2. Mucus hydrators

- **Hypertonic Saline—**Given the role of mucus dehydration to the pathogenesis of CF lung disease, rehydration can be achieved by osmotic agents, such as hypertonic saline (HS). The beneficial effects HS on mucociliary clearance were first identified in 1997 [47]. However, it was only in 2006 that HS was evaluated for its long-term efficacy by the National Hypertonic Saline Study Group [47]. Subsequently, many studies showed that this therapy significantly reduces exacerbation and mildly increases lung function [48–50].

In addition, *in vitro* studies suggested that HS increases the mucus clearance rate in patients due to mucus dilution and osmotically active ASL expansion. Interestingly, these effects were found to be prolonged on hyper-concentrated mucus samples, likely due to increased osmotic forces of the concentrated mucus [51]. Based on these findings, HS is consolidated as an additional therapy shown to be effective and inexpensive for patients. Nevertheless, studies have shown that HS has a short lifetime in the airways due to sodium absorption via ENaC [48,51,52]. Therefore, the development of other therapies with a prolonged effect would be of interest; for instance, combining HS with ENaC inhibitors could extend the

hydration benefit as a result of reduced ENaC-mediated ASL fluid reabsorption elicited by the inhalation of HS [51]. The net effect would be an increase in the duration of mucus hydration compared to HS alone [51]. Nevertheless, future clinical studies are needed to clarify if airway mucosal hydration is enhanced by the combined therapies [53].

- **Mannitol—**Several studies suggested that HS may inactivate important cationic antimicrobial defensins and contribute to infections and inflammation due to its ionic constitution [54–56]. Therefore, as an alternative non-ionic osmotic agent, mannitol was tested and approved in several countries as a dry inhalation powder named Bronchitol, which has been subsequently approved in the US in 2020 [57,58].

In comparison to HS, Branchitol's use reduces treatment burden and microbial contamination of nebulizers, as it is administrated via a small and portable dry-powder inhaler. Bronchitol safety and efficacy were evaluated in various phase 3 studies over the course of 26 weeks. The studies demonstrated that it improved the patient's forced expiratory volume in one second (FEV_1) when compared to the placebo group (NCT00630812, NCT00446680, NCT02134353). Although the drug was found to be safe, patients must do a Bronchitol tolerance test before starting treatment, as some can develop an allergic reaction [58–61].

3.3. CFTR Modulators

Small-molecule CFTR modulator drugs either correct and/or potentiate the CFTR protein by modulating its functioning. Correctors fix the defective folding of the protein and rescue its trafficking to the plasmatic membrane, while potentiators allow enhanced anion transport via CFTR by increasing this channel open probability [62–64]. The net result is a restoration of CFTR-mediated fluid secretion, reduction in mucus concentration, and reduced mucus viscoelastic properties. In the past few years, the ability to correct/modulate CFTR by different compounds has shown great promise to partially restore defective chloride and bicarbonate transport in CF cells and significantly improve CF patients' lung function [65].

The most recently approved modulator was Trikafta (Ivacaftor, Tezacaftor, and Elexacaftor) in 2019, which made CFTR modulators approved for 90% of the CF population with specific CFTR mutations [66–69]. A recent *in vitro* study pointed to a reduction in mucus concentration in response to Trikafta [70], but additional studies are necessary to address the long-term effects of CFTR modulators on mucus and mucociliary clearance restoration. The possible combination of modulators and mucus-targeting therapies can be even more relevant for CF adult patients with established lung disease and bronchiectasis. In addition, because ~ 10% of the CF population is still not eligible for current CFTR modulator therapy, there is an unmet need for new therapies for these patients [71].

4. Future perspectives

4.1. Reducing agents/mucolytics

As mentioned above, CF sputum has higher oxidative stress due to airway inflammation which generates mucin cross-link via multiple intermolecular disulfide bonds. Therefore,

researchers have pursued the development of disulfide bond reducing agents as a mucolytic therapy for many decades [28,49].

In the 1960s, Mucomyst (N-acetylcysteine [NAC]) started being used to treat CF; however, later clinical research demonstrated a limited effect of this drug in addition to adverse effects, such as bronchospasm and off-targeting irritation effect. Different studies highlighted that the NAC failure as a drug was due to its rapid transepithelial absorption and low intrinsic activity at neutral pH [72–74].

To compensate for the adverse effects, a more recent paper tested a novel reducing agent named “P3001” (Parion Sciences) that has a longer residence time and activity on the airway surfaces. The authors *in vitro* studies revealed that this compound is more effective than NAC and DNAse in reducing CF sputum viscoelasticity [73].

4.2. Inhibition of mucin production/secretion

In healthy airways, mucin production and secretion are essential to maintaining the ciliary clearance of harmful microorganisms. However, in CF, it is documented that the constant presence of airways insults leads to a detrimental upregulated synthesis and secretion of mucins [75,76]. Jaramillo et al., 2018, highlighted that, despite functionally interacting, the mucin synthesis and secretion processes involve different and complex pathways that have been studied as potential therapeutical targets [77].

Our group has focused on the proinflammatory IL-1 cytokines pathway as it has been observed that such cytokines are highly expressed in the supernatant of mucopurulent material (SMM) from the airways of excised CF lungs [78,79] and have been linked with CF airway inflammatory responses and mucus obstruction [80]. In agreement with these findings, Chen et al. demonstrated that IL-1β increases the production and secretion of MUC5B and MUC5AC in normal and CF HBE, with the predominant effect being MUC5B up-regulation [80]. Furthermore, the studies by Chen et al. demonstrated that mucin production was blunted in mice lacking either IL-1R1 or SAM Pointed Domain Containing ETS Transcription Factor (SPDEF), a mediator of Muc5b expression. Another study showed that the ER stress transducer IRE1b, a key mediator of airway mucin production and whose expression is up-regulated in CF, was decreased in *Spdef*$^{-/-}$ mice treated with IL-1β [81]. These findings indicate that IL-1β up-regulates MUC5B production via SPDEF-IRE1β signaling, suggesting that this pathway can be a therapeutic target for the hyper-concentrated CF mucus.

Recent research by our group indicated that the small molecule KIRA6 (kinase inhibiting RNase attenuator 6) decreases SMM-up-regulated IRE1α-dependent generation of the spliced form of the transcription factor XBP-1, which promotes cytokine production, including IL-1β, in CF airways [82]. Together, these findings suggest that the IRE1α/XBP-1 pathway may also be a therapeutic target for the hyper-concentrated CF mucus due to its functional role in IL-1β production. Decreasing the burden on the CF lung resulting from inflammatory mediator-increased mucus production is expected to be therapeutically beneficial, especially if combined with other therapies such as mucolytic agents.

4.3. Genetic therapies

Genetic therapies for cystic fibrosis have been in the spotlight since the CFTR gene identification in 1989. Like CFTR modulator therapy, gene therapies could treat the underlying cause of CF and, therefore, indirectly have a positive effect on CF mucus properties. Since the initial trials, the main challenges have been the delivery of the corrected CFTR gene and its persistence in the airways [83,84]. Nevertheless, the scientific knowledge about CF disease and the use of new models and technologies have improved drastically over the years. For example, *in vitro* models, such as human epithelial organoids and air-liquid interface lung cells, and animal models, such as CF ferrets and pigs, are essential to access the physiological effects of genetic therapies [85–87]. Furthermore, to improve the delivery of the corrected CFTR gene, other technologies developed were new viral and non-viral approaches such as reprogrammed AAV and lipid nanoparticles. CRISPR gene-editing, mRNA and genetically corrected airway stem cells are also growing prospects that are rapidly changing the field and broadening possibilities for a successful CF gene therapy on the horizon [88–90].

It is also important to note that many of the gene therapies in development focus on an inhaled delivery form, which can directly benefit the main organ affected by CF, the lungs. However, to successfully deliver gene therapy vectors to the airways, these inhaled genetic therapies have the challenge of overcoming a dense glycocalyx (tethered mucins) in the PCL, in addition to a viscoelastic mucus layer exacerbated in CF [91,92]. To this end, new nanoparticles (NPs) and viruses capable of penetrating the mucus barrier are being tested [93–95]. For example, *Wan et al*. showed that polymeric NPs combined with highly integrated lipid shells could significantly increase NPs penetration within the mucus layer [96]. In addition, it has been suggested that pretreatment with agents that can help hydrate mucus (i.e, mannitol, or HS) and/or cleave mucins (i.e., NAC) could enhance inhaled genetic therapies delivery efficiency [97]. Hence, further studies with mucus-targeting agents utilizing translational *in-vitro* models that mimic the CF airways mucus properties will be necessary to provide a proof-of-principle for these therapeutics approaches.

5. Conclusion

This review highlights that the abnormal mucus in CF airways is a key pathological factor, which is already observed in the early stages of CF lung disease. Therefore, studies on mucus cell biology, production, and biophysical properties in normal and CF airways are essential. Furthermore, this knowledge is promising for developing novel approaches in genetic therapy, small molecule inhibition of signaling pathways, CFTR modulation, and mucolytics. Therefore, tackling the mucus defects in CF is critical for successfully developing better therapies beneficial to all CF patients.

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Indications for structural and functional distinction from MUC5B. Proc Natl Acad Sci U S A 2021, 118.**This paper brings insights into mucin protein structure, which can open pathways for developing targeted therapies. The main finding was a distinct N- terminal domain in the MUC5AC protein that may explain why this mucin forms a more static and “tethered” mucus layer when compared to MUC5B.

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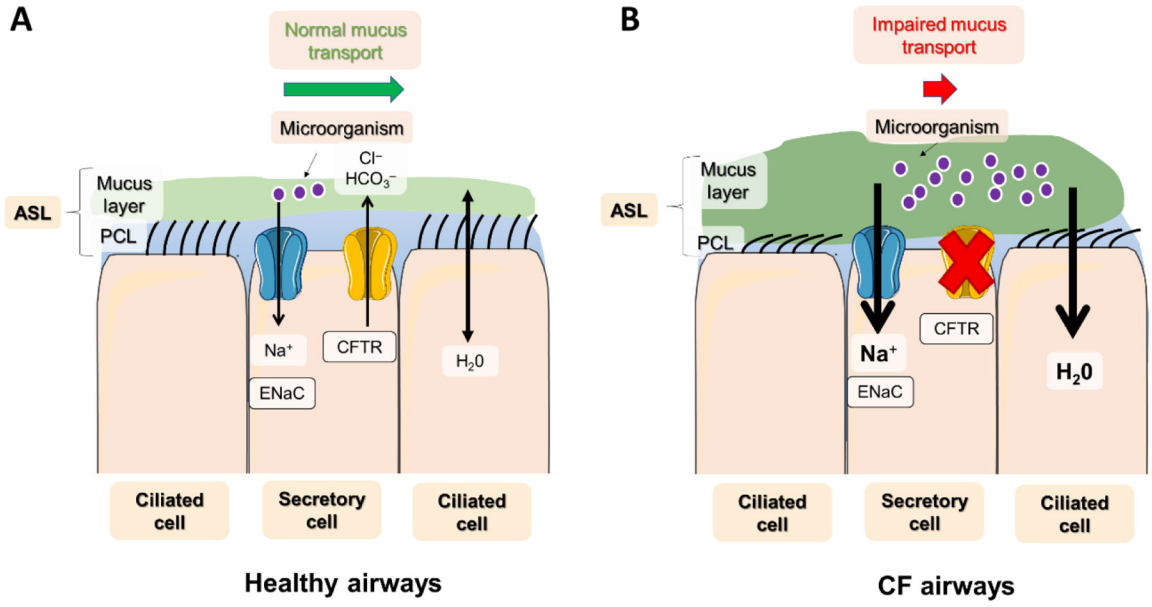

Figure 1: Mucus transport in healthy airways vs. CF airways.

A) In healthy airways, a well-balanced activity of ions (Na^+ and Cl^-), water, and mucus, across the lung epithelium results in an efficient mucus transport: Here, a well-hydrated PCL layer ensures that cilia can efficiently sweep away mucus and noxious inhaled microorganisms, preventing their accumulation in the airways. **B)** In CF airways, defective CFTR (mainly expressed in secretory cells) leads to impaired fluid secretion activity in mucin-producing goblet cells, leading to ASL dehydration and a PCL and cilia collapse that fails to transport mucus and infectious particles out of the lungs. The result is mucus obstruction and chronic bacterial infection in the airways and progressive lung disease.