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Effects of airway surface liquid pH on host defense in cystic fibrosis

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Abstract

Cystic fibrosis is a lethal genetic disorder characterized by viscous mucus and bacterial colonization of the airways. Airway surface liquid represents a first line of pulmonary defense. Studies in humans and animal models of cystic fibrosis indicate that the pH of airway surface liquid is reduced in the absence of cystic fibrosis transmembrane conductance regulator function. Many aspects of the innate host defense system of the airways are pH sensitive, including antimicrobial peptide/protein activity, the rheological properties of secreted mucins, mucociliary clearance, and the activity of proteases. This review will focus on how changes in airway surface liquid pH may contribute to the host defense defect in cystic fibrosis soon after birth. Understanding how changes in pH impact mucosal immunity may lead to new therapies that can modify the airway surface liquid environment, improve airway defenses, and alter the disease course.

Keywords

Cystic Fibrosis; antimicrobials; airway surface liquid; ASL

Introduction

Cystic Fibrosis (CF) is an autosomal recessive genetic disorder that affects multiple organs including the sweat duct, lungs, intestines, pancreas, and liver. The primary cause of morbidity and death in CF is progressive lung disease caused by chronic bacterial infection and inflammation. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene which encodes an anion channel regulated by nucleotide binding and cAMP-mediated phosphorylation. *CFTR* is localized to the apical membrane of cells of the surface airway epithelium and submucosal glands. It is especially abundant in ciliated cells (Kreda et al., 2005). In the lung, *CFTR* conducts HCO_3^- and Cl^- thereby

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helping regulate airway surface liquid (ASL) volume and composition. Loss of CFTR function has been implicated in an airways host defense defect, leading to impaired innate immunity and chronic bacterial colonization of the airways.

In the respiratory tract, ASL is a first line of defense against inhaled or aspirated pathogens including bacteria, fungi, and respiratory viruses. ASL comprises two layers: an aqueous (sol) layer and a mucus (gel) layer (Figure 1). The aqueous periciliary layer covers the cilia, hydrating mucins and allowing for ciliary beating by distancing the mucus from the cell surface. The mucus layer is comprised of secreted and tethered mucins produced by surface goblet cells and submucosal gland epithelia. This material traps inhaled and aspirated microbes so they can be removed from the lung via mucociliary clearance. In addition to trapping pathogens, ASL also contains numerous antimicrobial peptides, proteins, and lipids, the secreted products of surface and submucosal gland epithelia and resident phagocytic cells (Bartlett and McCray 2013).

While it has traditionally been thought that babies with CF are born with normal lungs, growing evidence indicates airway defenses are compromised early, perhaps as early as the first month (Khan et al., 1995; Armstrong et al., 1998). This defect contributes to lung disease progression during the first years of life and is characterized by colonization with bacteria (e.g. *Haemophilus influenzae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*), and the onset of inflammation. In addition to difficulties with bacterial infections, infants with CF are more likely to suffer greater morbidity from common respiratory virus infections, though the total number of viral infections is not different from non-CF (Abman et al., 1988; Hiatt et al., 1999).

The availability of new CF animal models, including the pig (Rogers et al., 2008) and ferret (Sun et al., 2008), has facilitated study of the early events of CF lung disease at the molecular and cellular level. While newborn CF pigs do not have pulmonary inflammation (Rogers et al., 2008), they exhibit an impaired ability to eradicate bacteria compared to their non-CF littermates (Stoltz et al., 2010). BAL and lung tissues removed from newborn CF pigs were less likely to be sterile than non-CF samples from non-CF littermates. CF pigs also exhibited a reduced ability to clear *S. aureus* when challenged via aerosol. A recent study by Pezzulo *et al* indicates the CF host defense defect in newborns is caused, in part, by abnormal ASL pH (Pezzulo et al, 2012). These studies showed that a reduction in pH leads to decreased ASL antimicrobial activity in CF pigs (Pezzulo et al, 2012), though the mechanism(s) by which pH impair ASL antimicrobials is currently unknown. The CF ferret also demonstrates early abnormalities in host defense, with tracheal xenographs from newborn CF ferrets exhibiting defective cAMP-induced chloride permeability and decreased submucosal gland fluid secretion (Sun et al., 2010). Juvenile and adult CF ferrets have decreased mucociliary clearance, increased mucus obstruction, and increased bacterial infections compared to non-CF littermates (Sun et al., 2013).

The pathogenesis of CF lung disease is complex and changes as patients age. As the disease progresses, the secondary complications of chronic inflammation and the protease rich environment of the airways further compromise host defenses. In this review we focus primarily on early events linked to the CF host defense defect and the onset of lung disease.

Pathogenesis

The role of ASL in the defenses of the airways

ASL plays a key role in the initial defense of the airways from pathogens. In addition to acting as a physical barrier to infection, ASL contains a number of peptide and protein antimicrobials. Some of the antimicrobials found in ASL include LL-37, lactoferrin, lysozyme, β -defensins, secretory leukocyte peptidase inhibitor (SLPI), and surfactant proteins A and D (SPA and SP-D). Many of these proteins possess both antibacterial and antiviral activity. Some phagocytic cells of the innate immune system, including macrophages and neutrophils, are also found in ASL.

CFTR helps regulate ASL pH

CFTR conducts chloride (Cl^-), bicarbonate (HCO_3^-) (Smith and Welsh, 1992), thiocyanate (SCN^-) (Pedemonte et al., 2007), and other anions. Of note, HCO_3^- secretion by airway epithelia helps regulate ASL pH. Loss of CFTR impairs HCO_3^- secretion in CF and leads to a decreased ASL pH (Coakley et al., 2003). A lower pH for CF versus non-CF has been reported for ASL removed from human primary airway epithelial cells (Coakley et al., 2003), cultured submucosal glands (Song et al., 2006), exhaled breath condensate from human patients (Tate et al., 2002), and tracheal ASL from newborn CF pigs (Pezzulo et al., 2012). The differences in ASL pH for non-CF and CF subjects may also depend on age and disease state. McShane and coworkers observed no differences in ASL pH between people with CF and non-CF controls aged 3 years or older (McShane et al., 2003). More recently, Abou Alaiwa and colleagues found that neonates with CF had a lower nasal ASL pH compared to non-CF neonates, whereas nasal pH in older CF children and adults was similar to values measured in people without CF (Abou Alaiwa et al., 2014). Further studies in CF animal models may aid in understanding how and when changes in ASL pH occur and how such changes influence the onset and progression of CF lung disease. Further work in this area may also aid our understanding of how changes in ASL pH might contribute to other respiratory diseases. For example, asthmatic subjects have been reported to have a reduced breath condensate pH compared to healthy controls (Brunetti et al., 2006; Antus et al., 2010).

Impact of pH on the activity of ASL antimicrobials

Antimicrobial peptides and proteins are key components of the innate immune response that help determine whether exposure to a pathogen leads to a disease state. Due to their ever ready, non-specific, and redundant nature, antimicrobials interact with microbes within minutes to hours of infection compared to the days or weeks required to mount an adaptive immune response. The CF pig model has demonstrated that an airway innate immune defect is present at birth (Stoltz et al., 2010; Pezzulo et al., 2012). Despite the presence of this defect, no difference in the abundance of key airway antimicrobials including lysozyme, lactoferrin, and SP-A was observed in newborn CF pigs (Pezzulo et al., 2012), suggesting that the animals' susceptibility to bacterial infections lies in an adverse ASL environment rather than the abundance of these proteins.

Changes in pH can have a variety of effects on protein function. The reduced pH of CF ASL is predicted to negatively impact the activity of many antimicrobials. For example, in an acidic environment the cathelicidin, LL-37, undergoes a conformation change that decreases its antibacterial activity (Johansson et al., 1998). SLPI (McNeely et al., 1997) and human neutrophil peptide 1 (HNP-1) (Daher et al., 1986) both possess antiviral activity that decreases as pH is lowered. SP-D and SP-A can inhibit *E. coli* growth at pH 6.0 and 7.4 but not at pH 5.0 (Wu et al., 2003). An acidic environment also reduces the amount of human β -defensin 1 produced by airway epithelial cells (Nakayama et al., 2002). Lactoferrin is not as pH sensitive as other antimicrobials in the airways and can chelate iron over a broad pH range down to pH 3.0 (Mazyrier and Spik, 1980). The lytic activity of lysozyme is stable between pH 5.8 and 9.3 (Davies et al., 1969). We note that lactoferrin displays antimicrobial activity independent of its iron binding capacity, and that lysozyme also displays muramidase-independent antimicrobial activity. Further research is required to determine if these other antimicrobial properties are pH-dependent. In non-CF pigs, lowering the pH of tracheal ASL reduced bacterial killing, while raising the pH of tracheal ASL in CF pigs improved bacterial killing (Pezzulo et al., 2012). The pH sensitivity of numerous airway antimicrobials is a probable causal link between reduced ASL pH in CF and the impaired antibacterial activity of CF ASL. Since the activity of ASL antimicrobials may be additive or synergistic (Singh et al., 2000), changes in pH may disrupt these cooperative interactions.

Mucin viscosity is pH dependent

While secreted and tethered mucins are key factors in airway defense, the abnormally viscous mucus found in the CF airways impairs mucociliary clearance and contributes to airway obstruction and bacterial colonization. Mucins are large, anionic, polymeric glycoproteins that are the primary component of mucus. Their abundance increases in response to inflammatory stimuli and remodeling of the airway epithelium (Bansil and Turner, 2006). Of note, mucins become more viscous at acidic pH (Bhaskar et al., 1991; Bansil and Turner, 2006), and this may further contribute to the thick mucus that characterizes CF lung disease. Jayaraman and coauthors reported that CF submucosal gland secretions are more viscous than non-CF secretions, though they did not detect a difference in pH between CF and non-CF secretions (Jayaraman et al., 2001). Recently, Gustafsson and colleagues reported that the addition of HCO_3^- to mucus from the small intestines of CF mice reduces both mucus density and its adherence to intestinal epithelium (Gustafsson et al., 2012). This is consistent with previous data from Chen *et al* that demonstrated that HCO_3^- reduces aggregation of porcine gastric mucins (Chen et al., 2010).

In addition to their role in mucociliary clearance, mucins also physically interact with antimicrobials. LL-37, a positively charged cationic peptide, binds to negatively charged molecules and macromolecules including mucins (Felgenreiff et al., 2006), DNA, and F-actin bundles (Bucki et al., 2007). Binding of LL-37 to these molecules leads to decreased antibacterial activity (Felgentreff et al., 2006; Bucki et al., 2007). Due to the direct interaction of LL-37 with mucins, it can be hypothesized that changes in the physical properties of mucins may alter the antimicrobial activity of LL-37. The increased abundance and viscosity of mucins of the CF airways may trap LL-37 and therefore decrease its availability to interact with microbes.

Other cationic antimicrobials that may interact similarly with mucins include the α - and β -defensins, lactoferrin, lysozyme, and SLPI.

The impact of reduced ASL pH on other aspects of host defense

ASL pH may also influence other components of airway defenses. There is evidence that reductions in pH lead to reduced ciliary beat frequency in human airways (Luk and Dulfano, 1983; Clary-Meinesz et al., 1998). While the mechanism behind this is not fully understood, pH has recently been linked to the regulation of ASL volume, with a reduction in pH favoring a reduced ASL volume (Garland et al., 2013). Therefore, in CF, a reduction in ASL volume may impair ciliary beating; the altered viscosity of CF mucus may further reduce the ability of cilia to clear mucus from the airways (Luk and Dulfano, 1983). Impaired mucus clearance provides a nidus for bacteria to remain in the airways, replicate, and colonize.

Additionally, clearance of bacteria from the CF airways may be reduced by impaired phagocyte function. *Ex vivo*, alveolar macrophage phagocytic activity decreases as extracellular pH is reduced (Schlesinger et al., 1992; Bidani and Heming, 1995). Activated alveolar macrophages also release reduced amounts of TNF- α (Bidani and Heming, 1995) and reactive oxygen species (ROS) (Bidani and Heming, 1995; Bidani et al., 2000) when cultured at lower pH values. It is currently uncertain whether or not CF macrophages have intrinsically altered bactericidal activity. A recent study demonstrated that when cultured under identical conditions, human CF and non-CF alveolar macrophages do not differ in their bactericidal activity against *P. aeruginosa* or in their intracellular ROS production (Cifani et al., 2013). However, earlier studies reported that monocyte derived macrophages (MDMs) from CF patients (Del Porto et al., 2011) and alveolar macrophages from CF mice (Di et al., 2006) possess decreased bactericidal activity compared to non-CF macrophages. The study by Di et al also showed deficient phagosome and lysosome acidification in murine CF alveolar macrophages (Di et al., 2006). However, since CF mice do not develop spontaneous lung disease similar to humans (Grubb and Boucher, 1999), it is difficult to reconcile findings in mice with the disease phenotype found in humans. Variations in the techniques used to isolate macrophages in the two human studies may also have contributed to the different findings. In addition, neither of the studies done with human samples address whether CF macrophages have impaired antimicrobial function prior to the onset of chronic lung disease, as both studies isolated macrophages from adult patients. Repeating these studies with macrophages from the CF pig and ferret models may shed light on whether or not there is an intrinsic defect in CF macrophage function and whether the environment of the CF airways, including reduced ASL pH, could reduce macrophage or neutrophil phagocytic activity or ROS production.

As CF lung disease advances, neutrophil-dominated inflammation is established in the airways. Proteases released from neutrophils and epithelia perturb the protease-antiprotease balance (Voynow et al., 2008). This imbalance further contributes to the CF host defense defect by promoting increased secretion of mucins and the degradation of antimicrobials (Garcia-Verdugo et al., 2010). Some proteases, such as cathepsins, are activated by acidic pH and proteolytically cleave antimicrobials, including lactoferrin and lysozyme (Britigan et

al., 1993), human β -defensins (Taggart et al., 2003), and SP-A (Lecaille et al., 2013), reducing their antimicrobial activity.

The airways have a complex host defense system that includes cough, the barrier properties of the epithelium, secreted mucus and antimicrobials, mucociliary clearance, and phagocytic cells. While changes in ASL pH are unlikely the sole explanation for the increased susceptibility of the CF airways to bacterial infection and colonization, it is a likely contributor to the early host defense defect. As discussed in this review, reduced ASL pH can increase the viscosity of mucus (Bhaskar et al., 1991; Bansil and Turner, 2006), inhibit the activity of some endogenous antimicrobials (Daher et al., 1986; McNeely et al., 1997; Johansson et al., 1998; Wu et al., 2003), decrease ciliary beat frequency (Clary-Meinesz et al., 1998), and impair phagocytic cell function (Schlesinger et al., 1992; Bidani and Heming, 1995). Each of these factors contributes to an airway environment in which bacteria are more likely to survive. The connections between loss of CFTR, reduced ASL pH, and the CF host defense defect provide a new therapeutic target for reducing the morbidity associated with CF lung disease.

Therapy

The work of Pezzulo *et al.* in the CF pig model suggests that increasing ASL pH may prevent or reduce airway infections (Pezzulo et al. 2012). A phase 2 clinical trial from 2006 reported that patients administered inhaled HCO_3^- expectorated three times more mucus than those given inhaled saline alone ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00177645) NCT00177645). However, to the best of our knowledge no published clinical studies demonstrate benefits of inhaled HCO_3^- or other pH-altering interventions in the treatment of early or established CF lung disease. It is possible that the pH change associated with aerosolized HCO_3^- may be too short lived to be therapeutically useful.

It may be feasible to modulate the pH of ASL by activating other HCO_3^- transport pathways or by inhibiting proton secretion (Figure 2). Further research will be needed to design and test drugs or small molecules that specifically increase HCO_3^- transport or inhibit proton secretion in the airways. Shamsuddin and Quinton reported Ca^{2+} -mediated HCO_3^- secretion in the small airways. This HCO_3^- secretion occurs independently of the cAMP-mediated secretion via CFTR and may be mediated by Ca^{2+} -activated chloride channels (CaCC) (Shamsuddin and Quinton, 2013). Pendrin, a $\text{Cl}^-/\text{HCO}_3^-$ exchanger, is another potential target for stimulating CFTR-independent HCO_3^- secretion (Garnett et al., 2011). Activating CaCC or pendrin in people with CF might stimulate sufficient HCO_3^- secretion to increase the ASL pH and improve host defense. Another approach to alter ASL pH is by inhibiting H^+ secretion, including H^+ channels and ATP-driven H^+ pumps. Two ATP-dependent transporters, the H^+/K^+ -ATPase and the vacuolar-type H^+ -ATPase (V-ATPase), contribute to apical proton secretion in both CF and non-CF airway epithelial cells (Coakley et al., 2003; Fischer and Widdicombe, 2006). Proton channels such as HCVN1 are also expressed in airway epithelia (Iovannisci et al., 2010). It should be noted that any effect of pH modulation as a therapy is likely to depend on the disease state of the patient. It may be more beneficial to increase pH early in the disease course to prevent or limit initial colonization with microbes.

Another possible benefit of raising ASL pH in CF airways is improved host defense against viral infections. There is growing evidence that respiratory viruses contribute to pulmonary exacerbations and bacterial colonization in CF patients (Armstrong et al., 1998; Hiatt et al., 1999; Kieninger et al., 2013). Many of the pH-dependent mechanisms of airway host defense that target bacterial infections may also influence susceptibility to viral infections, including mucociliary clearance and antimicrobial peptide function. In addition to improving airway host defenses against bacterial infection, increasing the pH of CF airways may also augment antiviral defenses. However, we note that reductions in ASL pH might also change the infectivity of viruses by altering the structure of viral glycoproteins, cellular receptors, or post entry steps in replication. Further studies are needed to determine if raising ASL pH increases the activity of antiviral molecules enough to exert an antiviral effect.

Conclusion

Studies in human cells, BAL, and lung tissues and CF animal models provide convergent evidence that airway defenses are not normal at birth in CF. ASL from newborn CF pigs has impaired antibacterial activity compared to the ASL of non-CF littermates (Pezzulo et al., 2012). CF ASL is also more acidic than non-CF ASL (Tate et al., 2002; Coakley et al., 2003; Song et al., 2006; Pezzulo et al., 2012; Abou Alaiwa et al., 2014) and the antibacterial activity of ASL is pH-sensitive (Pezzulo et al., 2012). A reduced ASL pH negatively impacts the innate host defense of CF airways in multiple ways (Figure 3), suggesting that pH may represent a therapeutic target as a means to prevent or reduce CF lung disease.

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Figure 1. A simplified model of the airway epithelium

The airway epithelium has two compartments, the surface epithelium and the submucosal gland (SMG) epithelium. The airway surface epithelium includes ciliated and non-ciliated cells, goblet cells, and basal cells (not shown). SMG epithelium includes ciliated duct cells, mucous cells, and serous cells that secrete antimicrobial proteins. ASL provides a barrier between the epithelium and inspired air. ASL is composed of two layers, a mucus (gel) layer and periciliary liquid. The periciliary liquid covers the cilia, providing an environment for the beating of the cilia and ASL clearance when pathogens become trapped. Various antimicrobials are found in ASL.

Figure 2. Schematic of proteins contributing to acid and base transport across the apical membrane of the airway epithelium

H^+ and HCO_3^- secretion help regulate ASL pH. CFTR provides a major HCO_3^- conductance that is lost in CF. Other sources of HCO_3^- transport across the apical membrane of airway epithelial cells include Ca^{2+} -mediated HCO_3^- secretion (CaCC) and pendrin, a $\text{Cl}^-/\text{HCO}_3^-$ exchanger. H^+ channels, V-ATPase, and H^+/K^+ ATPase are three apical membrane proteins that contribute to the acidification of ASL. We note that basolateral transport mechanisms and the paracellular pathway are also important to these processes (not shown).

Figure 3. A scheme for how changes in ASL pH may influence CF pathogenesis

CF is caused by loss of CFTR function, an anion channel that conducts Cl^- and HCO_3^- . Loss of CFTR function results in decreased HCO_3^- conductance across airway epithelial cells leading to decreased ASL pH. Many antimicrobials have reduced activity at a low pH. Cilia beat frequency is also reduced at lower pH values and mucins increase in viscosity as pH falls, leading to decreased mucociliary clearance. Phagocytic cell function may also be reduced in environments with lower pH. This decrease in antimicrobial activity

subsequently contributes to respiratory infections in the CF airway, caused by both viral and bacterial pathogens.

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