



Mucins, Mucus, and Sputum*

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Normal airway mucus lines the epithelial surface and provides an important innate immune function by detoxifying noxious molecules and by trapping and removing pathogens and particulates from the airway via mucociliary clearance. The major macromolecular constituents of normal mucus, the mucin glycoproteins, are large, heavily glycosylated proteins with a defining feature of tandemly repeating sequences of amino acids rich in serine and threonine, the linkage sites for large carbohydrate structures. The mucins are composed of two major families: secreted mucins and membrane-associated mucins. Membrane-associated mucins have been reported to function as cell surface receptors for pathogens and to activate intracellular signaling pathways. The biochemical and cellular functions for secreted mucin glycoproteins have not been definitively assigned. In contrast to normal mucus, sputum production is the hallmark of chronic inflammatory airway diseases such as asthma, chronic bronchitis, and cystic fibrosis (CF). Sputum has altered macromolecular composition and biophysical properties which vary with disease, but unifying features are failure of mucociliary clearance, resulting in airway obstruction, and failure of innate immune properties. Mucin glycoprotein overproduction and hypersecretion are common features of chronic inflammatory airway disease, and this has been the underlying rationale to investigate the mechanisms of mucin gene regulation and mucin secretion. However, in some pathologic conditions such as CF, airway sputum contains little intact mucin and has increased content of several macromolecules including DNA, filamentous actin, lipids, and proteoglycans. This review will highlight the most recent insights on mucus biology in health and disease.

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Abbreviations: ASL = airway surface liquid; CF = cystic fibrosis; EGFR = epidermal growth factor receptor; F-actin = filamentous actin; FoxA2 = forkhead box A2; IL = interleukin; NF = nuclear factor; PG = prostaglandin; TLR = Toll-like receptor; TNF = tumor necrosis factor

Normal mucus and mucociliary clearance are critical components of lung innate immune function. Airway mucus maintains hydration in the airway and traps particulates, bacteria, and viruses. Mucus also has antioxidant, antiprotease, and antimicrobial activities. While the terms *sputum* and *mucus* are commonly used interchangeably by clini-

cians, these are very different substances. The gel forming mucins are the principal polymeric components of normal mucus, but this is rarely true in chronic airway disease. While mucin is generally cleared by cilia, with chronic inflammation the ciliated epithelium becomes damaged and the increased volume of secretions often requires clearance by cough. These expectorated secretions are called sputum.

The major macromolecular constituents of mucus are the mucin glycoproteins.¹ Mucins are large glycoproteins with a signature feature of tandemly

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repeating amino acids rich in serine and threonine, which are the sites for O-linked glycosylation.² Secreted mucins form large oligomeric structures that impart viscoelastic properties to mucus.¹ Importantly, mucin glycoproteins may also contribute important antimicrobial and anti-inflammatory properties.

For normal function, mucus must be cleared by ciliary motion and this process requires a balance between the volume and composition of the mucus, adequate periciliary liquid volume, and normal ciliary beat frequency.³ This balance may be stressed by inflammatory conditions that induce mucin overproduction and hypersecretion. However, in cystic fibrosis (CF) and other chronic inflammatory airways diseases, other large polymers predominate in the airway secretions; these polymers include DNA, filamentous actin, proteoglycans, and biofilms, which in combination with bacteria and inflammatory cells constitute sputum.⁴ Excessive sputum overwhelms ciliary clearance and obstructs airways, causing morbidity and mortality in chronic inflammatory lung diseases like asthma, CF, and COPD.

This review will highlight our current understanding of mucin gene regulation and regulation of mucin secretion. We will also discuss how mucus biology is perturbed in disease and how insights regarding the biochemical and biophysical characteristics of mucus and sputum need to be considered to more effectively treat airway obstruction in chronic inflammatory airway diseases.

MUCIN BIOLOGY

The mucin protein backbones are encoded by genes called *MUCs*. Many *MUC* genes are expressed in the airway including *MUC1*, *MUC2*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC7*, *MUC8*, *MUC11*, *MUC13*, *MUC15*, *MUC19*, and *MUC20*.² The protein structures encoded by these genes segregate into three major families: secreted, gel-forming mucins (predominantly *MUC5AC* and *MUC5B*, with smaller contributions from *MUC2*, *MUC8*, and *MUC19*); membrane-associated mucins that may function as cellular receptors (*MUC1*, *MUC4*, *MUC11*, *MUC13*, *MUC15*, *MUC20*); and a non-gel-forming, secreted mucin (*MUC7*). Mucins have been very difficult to study because of their large size and because attached sugars mask a large portion of the protein domains. Recently, antibodies have become commercially available to identify the protein backbones of *MUC1*, *MUC2*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC7* and *MUC8*. Using *MUC*-specific antibodies, investigators¹ have identified the major secreted mucin glycoproteins in human airway se-

cretions as *MUC5AC* and *MUC5B*; this corresponds with *in vitro* data obtained by analysis of mucin glycoproteins in secretions from primary cultures of human airway cells.⁵

The localization of mucins is restricted to specific compartments in the normal airway. The membrane-associated mucins, *MUC1* and *MUC4*, are present at the apical surface of ciliated cells. The secreted mucins, mainly *MUC5AC* and some *MUC5B*, are expressed in goblet cells, while *MUC5B* is the predominant mucin expressed in the mucous cells of the submucosal gland.^{1,6,7} *MUC7* is expressed in serous cells of the submucosal glands and expression levels are not altered in CF.⁸ In chronic inflammatory airway diseases, *MUC2* expression is detectable in the goblet cells,⁹ and *MUC5AC* and *MUC5B* expression is increased.⁶ Of the secreted mucins, *MUC2* was the first *MUC* gene identified in the lung, although *MUC5AC* and *MUC5B* appear to be major human airway mucin glycoproteins. In mouse models, *Muc5ac* is a marker of goblet-cell metaplasia.¹⁰ Therefore, *MUC2*, *MUC5AC* and *MUC5B* have been the most intensively studied *MUC* genes for evaluation of airway mucin gene regulation and mucin glycoprotein secretion. Figure 1 is a summary of known transcriptional signaling pathways regulating *MUC5AC*.

MUCIN GENE REGULATION

Consistent with their role in airway innate immunity, mucins are upregulated by pathogens, inflammatory mediators, and toxins that exacerbate chronic inflammatory lung diseases such as CF, COPD, and asthma.^{2,6} This field was pioneered by Dr. Carol Basbaum, who cloned the promoters for *MUC2* and *MUC5AC* and was the first to show that bacteria upregulated mucin gene expression.⁹ Her laboratory and others went on to show that many bacteria including *Pseudomonas aeruginosa*,^{9,11} *Staphylococcus aureus*,¹² *Streptococcus pneumoniae*,¹³ nontypeable *Haemophilus influenzae*,^{14,15} and *Mycoplasma pneumoniae*¹⁶ activate cell surface receptors resulting in nuclear factor (NF)- κ B activation and *MUC2* and/or *MUC5AC* transcriptional up-regulation.² Respiratory viruses also activate mucin expression in airway cells. Rhinovirus upregulates *MUC5AC* and *MUC5B* gene expression in airway epithelial cells,¹⁷ while other respiratory viruses including influenza,¹⁸ RSV,¹⁹ and paramyxoviruses²⁰ induce *Muc5ac* gene expression associated with mucous cell metaplasia in mice.

Several cytokines including tumor necrosis factor (TNF)- α ,²¹ interleukin (IL)-1 β ,²² and IL-13²³ up-regulate *MUC5AC* expression, and IL-6 and IL-17

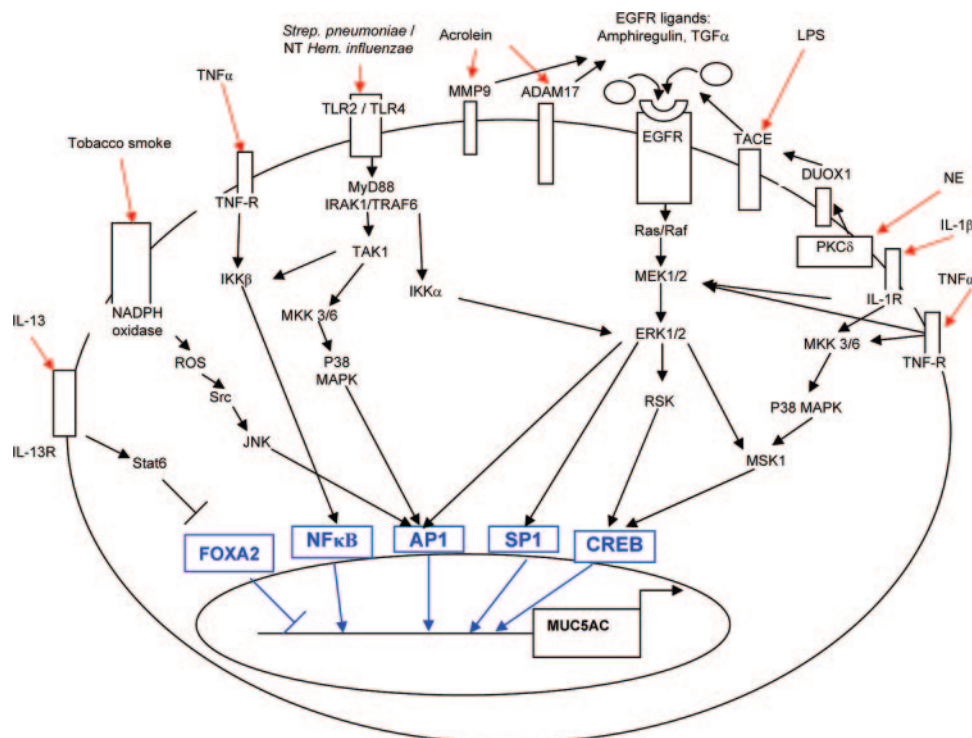


FIGURE 1. Regulation of *MUC5AC* gene transcription. The stimuli regulating *MUC5AC* in this diagram are limited to those with defined signaling pathways leading to transcriptional regulation. Stimuli are noted by red arrows. Transcription factors are in blue. Arrows denote positive signaling pathways. Both EGFR-dependent and EGFR-independent pathways activate *MUC5AC* transcription. Mitogen-activated-protein-kinase signaling pathways result in activation of several alternative transcription factors required for *MUC5AC* upregulation. AP1 = activator protein 1; CREB = cyclic adenosine monophosphate response element binding protein; ERK = extracellular signal-related kinase; IKK = I κ B kinase; IRAK1 = IL-1 receptor-associated kinase 1; MEK = mitogen-activated protein/extracellular signal-regulated kinase 1; MKK = mitogen-activated protein kinase kinase; MSK1 = mitogen- and stress-activated protein kinase 1; NE = neutrophil elastase; ROS = reactive oxygen species; RSK = p90 ribosomal S6 kinase; SP1 = specificity protein 1; TACE = TNF- α converting enzyme; TAK1 = transforming growth factor β -activated kinase 1; TRAF6 = TNF receptor-associated factor.

upregulate both *MUC5AC* and *MUC5B* expression.²⁴ IL-13, a central mediator of airway remodeling in asthma,²⁵ increases *MUC5AC* expression by several indirect mechanisms including upregulation of transforming growth factor- β ²⁶ and Stat6 phosphorylation and suppression of the transcription factor, forkhead box A2 (FoxA2).²³ FoxA2 inhibits *MUC5AC* expression and FoxA2 expression is inversely related to goblet-cell metaplasia.²⁷ Complement C3 upregulates murine *Muc5ac* *in vivo* and *in vitro*.²⁸ Airway proteases including neutrophil elastase,^{29–31} matrix metalloproteinase 9,³² tissue kallikrein,³³ and human airway trypsin³⁴ upregulate *MUC5AC* expression. Finally, pollutants and oxidants including prostaglandin (PG) E₂,³⁵ hydrogen peroxide,³⁶ dual oxidase 1,³⁷ tobacco smoke,³⁸ acrolein,³² and residual oil fly ash,³⁹ all upregulate *MUC5AC* messenger RNA expression. These stimuli activate several signaling pathways resulting in the activation of NF- κ B, activating protein 1, specificity

protein 1, or cyclic adenosine monophosphate response element binding protein promoter elements^{2,6} (Fig 1). Several stimuli activate epidermal growth factor receptor (EGFR)-mediated and alternative signaling, resulting in redundant pathways for *MUC5AC* upregulation.^{38,40} In contrast to the pro-inflammatory stimuli that increase *MUC* expression, the glucocorticoid dexamethasone is one of the few agents that transcriptionally decreases *MUC5AC* messenger RNA abundance in airway epithelial cells.⁴¹

Transcriptional regulation is likely not the only mechanism of *MUC* gene expression. Several inflammatory mediators, including neutrophil elastase, TNF α , and IL-8, increase *MUC5AC* steady state expression by increasing messenger RNA stability in human airway epithelia.² *MUC2* and *MUC5B* are regulated by DNA methylation and histone deacetylase activity in cancer epithelial cells, suggesting that epigenetic mechanisms of *MUC* regulation may play a role in inflammatory airway diseases.⁴²

REGULATION OF MUCIN SECRETION

In the healthy individual, circadian rhythms regulate normal submucosal gland mucus secretion, principally through the vagal nerve. However, in patients with inflammatory airway diseases, mucus hypersecretion from metaplastic and hyperplastic goblet cells likely contributes to mucus obstruction of airways.⁴³ Mucus secretion is stimulated by several inflammatory stimuli such as the following: (1) oxidants including tobacco smoke,⁴⁴ residual oil fly ash,⁴⁵ nitric oxide,⁴⁶ and superoxide via PGF2 α ⁴⁷; (2) proteases⁴⁸ including neutrophil elastase,^{49,50} and *P aeruginosa* proteases⁵¹; and (3) cytokines including TNF- α ⁵² and platelet activating factor.⁵³ Common signaling intermediates for many stimuli include G protein-coupled receptor activation, protein kinase C activation, tyrosine phosphorylation, and phospholipase activation.^{54,55} The molecular components of the mucin granule exocytic machinery are currently being determined.⁵⁶ The myristoylated alanine-rich C-kinase substrate protein is a component of the secretory complex required for mucin granule exocytosis. A peptide related to myristoylated alanine-rich C-kinase substrate inhibits stimulated exocytosis *in vivo*, suggesting a novel approach to regulate mucin hypersecretion.^{57,58} As the secretory machinery of mucin granules is defined, the proteins required for mucin exocytosis will provide new targets for therapies to control mucus hypersecretion.⁵⁹

MUCIN FUNCTION

The precise functions of specific mucin protein domains are not well understood. Most work has focused on the role of membrane-associated mucins as receptors and signaling molecules. MUC1 is a receptor for *P aeruginosa* flagellin.⁶⁰ Flagellin binding to MUC1 competes with flagellin binding to Toll-like receptor (TLR) 5, and therefore MUC1 inhibits flagellin-activated TLR-5-mediated signaling and IL-8 release.⁶¹ The MUC1 cytoplasmic tail participates in outside-inside signaling.⁶² Following ligand binding to the MUC1 extracellular domain, the MUC1 cytoplasmic tail interacts with Src family nonreceptor tyrosine kinases, resulting in activation of phospholipase C, protein kinase C, and downstream extracellular signal-related kinase 1/2. MUC1 has been reported to interact with ErbB family members, and components of the Wnt signaling pathway. Another major membrane-associated mucin, MUC4, has been reported to activate ErbB2 in colon cancer cells,⁶³ but this function has not yet been confirmed in the lung.

Functions for the various motifs in the protein domains of secreted mucins have been more difficult to elucidate. Muc2-null mice develop colitis or colon

carcinoma, suggesting that secreted mucins play an important role in preventing epithelial inflammation in the colon.^{64,65} Epidemiologic data associate MUC7 allele length polymorphisms with a propensity for asthma and decreased lung function with age,⁶⁶ although the functional impact of changes in tandem repeat sequence length is not known. Finally, several investigators^{67–70} have demonstrated that *P aeruginosa* binds to mucin carbohydrate structures including amino sugars and fucose. This function underlies the importance of normal mucus composition and efficient mucociliary clearance to rid the airways of pathogens and toxins.

MUCUS AND SPUTUM STRUCTURE AND BIOPHYSICAL PROPERTIES

Rheology is the study of the response of material to an applied deforming force. The rheology of airway secretions is determined by their polymer composition and structure and their interaction with cilia and airflow as deforming forces. Rheology is a dynamic rather than a static measurement; thus, rheologic testing of viscoelastic substances like mucous gels yields different results with different applied stresses. In the studies of the rheology of airway secretions, forces are usually chosen to mimic the low-stress, high-frequency forces applied by beating cilia; the low-stress, low-frequency extrusion of secretions from glands; or the high-velocity, high-stress forces imposed by cough airflow. Classic rheologic measurements include the following: viscosity or energy loss and deformation; elasticity or energy storage; mechanical impedance or rigidity as the absolute resistance to deformation; shear thickening or thinning; reversible shear thinning (thixotropy); and apparent yield stress, which is a catastrophic decrease in viscosity when polymeric bonds are irreversibly ruptured. Each of these may have a role in the behavior of airway secretions.⁷¹

Mucus polymer structure has been characterized as a tangled network. Mucins oligomerize into long linear chains similar to railcars hooking up on a long freight train.¹ It is thought that disulfide bond cross linking is inhibited by mucin glycosylation but that these elongated oligomers will tangle to form a low viscosity network. Recognition that in some diseases, such as severe asthma, there is indeed intermolecular cross linking between mucin chains suggests that this tangled network structure may be more physically complex under some circumstances.⁷² This is also seen in plastic bronchitis (Fig 2, right, C). Although it is assumed that each of the elongated mucin oligomers is made of a single mucin species (primarily MUC5AC or MUC5B), it is possible that these are heteromeric and this would suggest further polymerization after exocytosis.

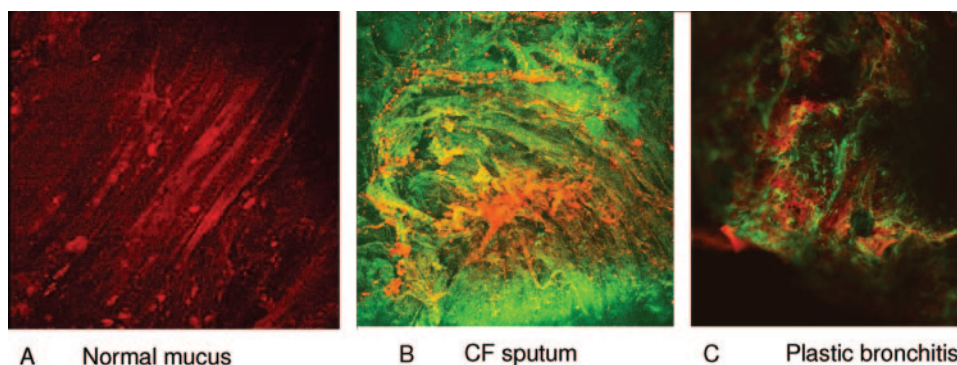


FIGURE 2. Laser scanning confocal micrograph of mucus and sputum. Normal mucus (*left, A*), cystic fibrosis sputum (*center, B*), and a plastic bronchitis secretion plug (cast) [*right, C*] are shown (unpublished data). Mucin is stained with Texas red phalloidin conjugated to UEA lectin (red) and DNA with YOYO-1 (green). In plastic bronchitis secretions (*right, C*), the mucin stain shows a complex cross-linked polymer that is different from the linear polymers seen in normal airway mucus (*left, A*), and the DNA is only on the outside of the cast in discrete cells or short polymers, suggesting a secondary inflammatory response. In contrast, in CF sputum (*center, B*), there is a paucity of mucin staining, and DNA polymers are present in long linear bundles. All images are 200×200 microns.

The secondary polymer structure consisting of DNA (Fig 2, *center, B*) and filamentous actin (F-actin) copolymers is more rigid, consisting of thicker polymer bundles that do not coassociate with the mucin network. This secondary network is characteristic of sputum or pus. It tends to have a lower elasticity than pure mucin and is the result of inflammatory cell necrosis. This is the dominant polymeric species in diseases like CF and bronchiectasis, and it changes the rheologic properties of the secretion.⁷³ Therapies that target DNA polymers (deoxyribonuclease) and F-filamentous actin polymers (thymosin $\beta 4$), may decrease CF sputum viscoelasticity.⁷⁴

Cough and ciliary clearance are also highly dependent on the interaction between the epithelial or ciliary surface and the surface of the secretion. Surface interactions are nearly independent of polymeric structure. Surface forces are dominated by surface tension and surfactant interactions that are characterized by wettability and interfacial tension (Fig 3). Assuming a sessile drop, Young's equation allows the calculation of adhesive work determined as $\gamma(1 + \cos \theta)$, where γ is the interfacial tension and θ is the contact angle at the solid-liquid-air interface. Combined with the intermolecular attractive force of cohesivity, this yields a measurement called tenacity; the greatest determinant of the cough clearability of secretions. Although the viscoelasticity of CF sputum is lower than that of either asthma or chronic bronchitis secretions, tenacity is greatly increased due to very high adhesivity.⁷⁵ Interfacial and adhesion tension is markedly increased by hydrolysis of the normal surfactant layer, which permits transfer of energy from the cilia to the mucus while preventing ciliary entanglement.⁷⁶⁻⁷⁸ Finally, *in vitro* stud-

ies⁷⁹ suggest that CF airway surface liquid is diminished when adenosine triphosphate is decreased in the airway surface liquid (ASL) and purinergic receptor P_2Y activation of alternative chloride is silenced. The loss of sufficient ASL may impede ciliary motion and results in mucus stasis.⁸⁰

MUCUS AND SPUTUM CLEARANCE

There must be an essential balance between adequate mucus production and mucus clearance for optimal airway defense. Airway mucus clearance can be modeled using equations common to fluid dynamics. Putting these mathematical models into common English, we can say that when inflow (mucus production) is high (hypersecretion) there can be overflow (expectoration) even with normal outflow (mucociliary clearance) and when outflow is impaired, even with normal or low inflow, there can be sludging that further exacerbates obstruction. It is likely that infection and irritants initially increase both the production and the clearance of secretions to augment airway defenses. However, when clearance is disturbed, as in patients with life-threatening asthma, there can be profound consequences with airway obstruction and gas trapping.

The rheologic properties that favor mucociliary clearance appear to hinder cough clearance and vice versa.⁸¹ The ciliary interaction requires that the cilia efficiently transmit energy to the mucus layer by elastic interaction, and that the cilia can effectively sweep through the mucus unhindered by high viscosity or by low-volume ASL. For airflow-dependent clearance, secretion viscosity and cohesivity allow the secre-

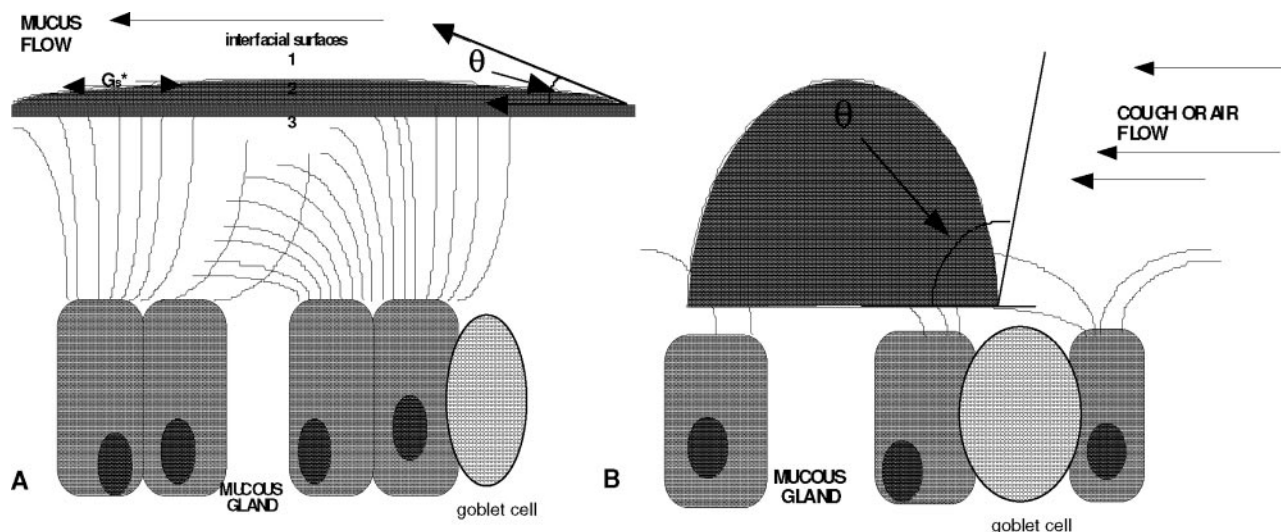


FIGURE 3. Model demonstrating the epithelial and mucus surface forces in health and disease. Source of mucus is from both goblet cells and submucosal glands. *Left, A:* The proposed effect of surface forces on mucus transport in the healthy airway. A surfactant layer may facilitate the mucus wetting (spreading over) the underlying epithelium after goblet-cell exocytosis or extrusion from submucosal glands. Increased wettability is measured by a small contact angle, θ , and decreased surface mechanical impedance G^* s. Interfacial tension $\gamma_{1,2}$ is measured at the air (1)-mucus (2) interface, and the contact angle θ is measured at the mucus (2)-periciliary/solid (3) interface. *Right, B:* The effect of surface forces on sputum cough transport in the diseased airway. Surfactant hydrolysis to lysophospholipids may decrease wettability and increase interfacial tension. Coupled with stimulated mucus hypersecretion, this leads to a buildup of mucus on the top of the epithelium.

tions to remain intact in the high-velocity airflow, a low elasticity prevents recoil, and apparent yield stress augments the bolus transport of secretions. It follows that medications that decrease viscosity, such as mucolytics, may benefit ciliary clearance but hamper cough clearance, while medications that decrease the adhesion of secretions to the epithelial surface are likely to improve airflow-dependent clearance. Alternatively, in CF, augmenting ASL volume and/or decreasing ASL viscosity by using hypertonic saline solution increases mucus clearance.⁸²

Although it has long been assumed that patients with CF have mucus hypersecretion and there appears to be stimulation of mucin gene expression in the CF airway, CF sputum contains almost no intact mucin.⁷³ This may be due to proteolytic degradation of mucins and accumulation of other biopolymers such as proteoglycans, DNA, lipids, and F-actin, which dilute the relative concentration of secreted mucins.⁴ During an acute exacerbation of CF when expectorated sputum volume increases, the mucin concentration in sputum also increases, suggesting that the cellular mechanisms for mucin protein production and exocytosis remain intact.⁸³ Regardless of the mechanism leading to decreased mucins in CF sputum, this reduction alone strongly suggests that we must be cautious in using medications meant to decrease mucin secretion in CF.

The critical protective role of mucins in preventing airway disease, the relationship between secreted

mucins, mucus, and sputum polymers, and the optimization of sputum clearance all require further exploration in order to develop appropriate therapy for specific airway diseases.

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