ORIGINAL ARTICLE

Hypertonic saline releases the attached small intestinal cystic fibrosis mucus

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SUMMARY

Hypertonic saline inhalation has become a cornerstone in the treatment of cystic fibrosis (CF), but its effect on CF mucus is still not understood. In CF, mucus stagnates in the airways, causing mucus plugging, and forming a substrate for bacterial invasion. Using horizontal Ussing-type chambers to allow easy access to the tissue, we have recently shown that the small intestinal mucus of CF mice is attached to the epithelium and not freely movable as opposed to normal mucus, thus pointing to a similarity between the CF mucus in the ileum and airways. In the same type of system, we investigated how hypertonic saline affects mucus thickness, attachment and penetrability to fluorescent beads the size of bacteria in ileal explants from the cystic fibrosis transmembrane conductance regulator mutant (ΔF508) mouse, in order to characterize how this common therapy affects mucus properties. Hypertonic saline (1.75–5%) detached the mucus from the epithelium, but the mucus remained impenetrable to beads the size of bacteria. This approach might be used to test other mucolytic interventions in CF.

Key words: cystic fibrosis, hypertonic saline solution, ileum, mucin, mucus.

INTRODUCTION

Cystic fibrosis (CF) is a recessive genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), where the most common mutation is a deletion of phenylalanine 508 (ΔF508).[1] The CFTR channel is a chloride channel localized to the apical membrane of epithelial cells.[2] More recently, it was found to also transport bicarbonate ions.[3] CF is characterized by tenacious mucus, most noticeably affecting the lungs, pancreas and the intestine.[4,5] It is only during recent years that the link between CFTR dysfunction and the altered mucus properties has started to be clarified. We have recently shown that sufficient bicarbonate at the site of mucus release in the small intestine is necessary for unpacking mucins. Thus, tissue lacking functional CFTR yields a dense mucus that is attached to the epithelium, something that can be compensated for by adding 100 mmol/L bicarbonate.[6] Correction of the dysfunctional CFTR channel causing CF is the ultimate goal in the effort to develop an efficient cure, a feat that has been accomplished recently for one particular and rare mutation, G551D.[7] However, for the vast majority of CF patients, this manner of therapy is not available, and it is more difficult to envision such specific therapies for all CF-causing mutations. Hence, other treatments not directly targeting CFTR malfunction must be studied and evaluated.

Current CF therapies are a combination of lung drainage methods, pancreatic enzyme replacement and an active antibiotic treatment strategy.[8] Recently, inhalation of hypertonic saline (HS) solutions has been introduced and widely used with good effects.[9,10] HS has been shown to improve lung function in CF patients, as evaluated in a number of clinical studies.[9,10] However, the mechanism for the HS effect on the mucus remains largely unknown, except for a few *in vitro* studies that suggest alternative mechanisms for the beneficial effect of this treatment.[10–12] We have developed an experimental method where mucus can be studied and its properties evaluated after secretion from ileal explants.[6] CF patients commonly have intestinal problems, including meconium ileus and distal intestinal obstruction syndrome. CF mice have a similar intestinal phenotype, which can be controlled by adding salts and polyethylene glycol (a mild laxative) to the drinking water, in conjunction with a liquid diet if required.[13] We have previously shown that for proper unfolding of the MUC2 mucin, the calcium and hydrogen ions bound to the mucin when packed in the goblet cell granule must be chelated on release.[14] This is accomplished by bicarbonate ions chelating calcium and increasing the pH. When this mechanism is compromised, as in CF, the mucin is insufficiently unfolded and is attached to the epithelium.[6] Using normal homozygous wild-type (WT) and CF (ΔF508, *Cftr*^{tm1eur}) mice, and a modified Ussing-type set up, we have now studied how HS affects CF mouse ileum mucus properties in terms of thickness, attachment and penetrability.

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RESULTS

Mucus in WT C57BL/6 mouse ileum forms a loose layer that is easily aspirated, whereas the mucus in the CF mouse is denser and difficult to aspirate.[6,15] The differences between the WT and CF mucus are clearly illustrated by a video showing the mucus formed on ileal tissue mounted in a horizontal Ussing-type chamber, where the otherwise transparent mucus is visualized by surface charcoal.[16] Initial and final frames from video recordings of WT are shown in Fig. 1a and Movie S1, and from a CF mouse in Fig. 1b and Movie S2. The WT mucus is easily removed, whereas CF mucus is attached to the tissue and can be moved around, but not aspirated.

To further show the difference between mucus in WT and CF mice, Carnoy-fixed paraffin-embedded sections were stained with the anti-MUC2-C3 antibody (green), directed against the main mucus component, the Muc2 mucin. In sections from WT mice, mucus is sparse in the crypts (Fig. 2); but in the sections from CF mice, mucus fills the crypts, making them appear dilated and clogged with mucus as reported previously for this mouse strain.[13] As we have previously shown, there are also more goblet cells per crypt in the CF mouse ileum.[6]

Because HS is an accepted treatment for CF lung disease, we wanted to know if and how mucus was affected in our experimental system. After the ileal tissue was mounted in the Ussing chamber and temperature equilibrated, the apical solution was replaced with increasing amounts of NaCl in normal Krebs' buffer, to make the solution hypertonic. After 1 h, during which tissue viability was constantly assessed by transepithelial PD recording, the mucus thickness was measured, mucus that could be aspirated was removed and the thickness of the remaining mucus measured. As shown previously, CF mucus secreted into physiological NaCl (0.9%) could not be aspirated in contrast to WT mucus, which could be aspirated (WT Fig. 3a and CF Fig. 3b).[6] Mannitol inhalations are also used to improve lung function in CF patients.[17] In our experimental set up, the attached CF mouse ileal mucus became easily aspirated after 1 h incubation with 70 mmol/L mannitol (Fig. 3a, compare 0.9% NaCl and 10 mmol/L mannitol in Fig. 3b). When the mannitol concentration was kept at 10 mmol/L, the CF mucus detachment was dependent on NaCl concentrations, and it was still not possible to aspirate CF mucus after incubation with 1.0% and 1.5% NaCl. However, when incubating with 1.75% and higher concentrations (up to 5%) of HS, the CF mucus could be aspirated (Fig. 3b). To further visualize this effect, a movie of CF explants incubated with 2% for 40 min was recorded (Fig. 1c; Movie S3). As shown, this treatment made it possible to aspirate the CF mucus, leaving the intervillus space almost as empty as in WT tissue.

As can be observed in Fig. 3b, the mucus thickness increased after exposure to HS. This increase in thickness is further shown in Fig. 3c. The mucus thickness did not increase significantly when incubated with 1%, 1.5% or 1.75% NaCl (170, 260 or 300 mmol/L, respectively). When the apical solution contained 2%, 3%, 4% or 5% NaCl (340, 510, 680 or 860 mmol/L, respectively) the mucus thickness before aspiration increased by 71 ± 12, 137 ± 62, 145 ± 50 and 90 ± 47 μm, respectively (Fig. 3c). Importantly, at 1.75% the mucus could be easily aspirated, and therefore the increase in mucus thickness did not correlate with this parameter under these conditions. Apical incubation with HS pulls water from the epithelial cells and causes cell shrinkage. It is likely that the increased mucus thickness is caused by liquid drawn out of the epithelial cells and into the mucus. The hypertonic solution also increased cell shedding from the villi tips, identified as small blebs in the mucus (Fig. 1c, right). This effect explains why the villi looked slightly deformed, but it should be noted that treatment did not cause complete disruption of the tissue. The transepithelial potential difference (PD) remained stable and normal up to 5% NaCl, aside

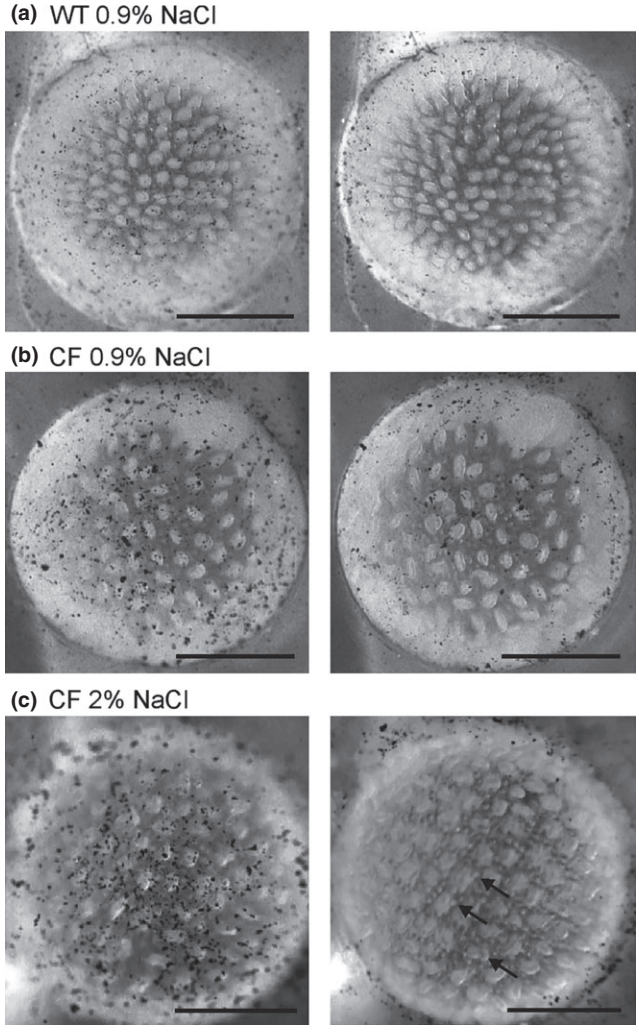

Fig. 1 Ileal mucus in cystic fibrosis (CF) mice was attached to the epithelium, but can be detached by treatment with hypertonic saline. (a) Movie (Movie S1) showing aspiration of easily removable normal homozygous wild-type (WT) mucus from an ileal explant mounted in the Ussing-type chamber. The first (left) and last (right) frames of the movie are shown in the printed manuscript. (b) Movie (Movie S2) showing attempt to aspirate the attached CF mucus from an ileal explant mounted in the Ussing-type chamber. The first (left) and last (right) frames of the movie are shown in the printed manuscript. Movies in (a) and (b) are representative of more than 10 animals of each genotype. (c) Movie (Movie S3) showing aspiration of CF mucus after treatment with 2% NaCl for 40 min from an ileal explant mounted in the Ussing-type chamber. The first (left) and last (right) frames of the movie are shown in the printed manuscript. Arrows in (right) indicate blebs formed from the villi tips after hypertonic saline incubation, although they are difficult to observe in 2-D images. Bar, 1 mm.

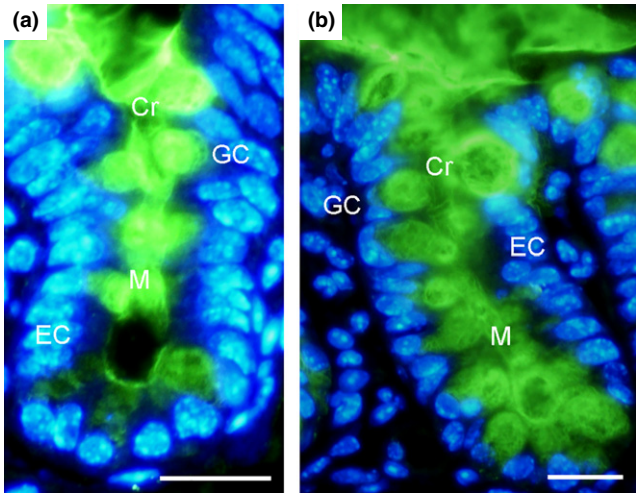

Fig. 2 Mucus in cystic fibrosis (CF) mouse ileum accumulated in the crypts. (a) Immunostaining of Muc2 (green) and nuclei (blue) in normal homozygous wild-type (WT) mouse ileal crypts. (b) Immunostaining of Muc2 (green) and nuclei (blue) in CF mouse ileal crypts. Bar, 20 μm, representative images of $n = 5$ in each group. Cr, crypt; EC, epithelial cell; GC, goblet cell; M, mucus.

from a transient dip concurrent with the addition of HS. However, at higher NaCl concentrations the normal PD was lost, reflecting loss of tissue viability.

The mucus in WT small intestine is normally penetrable to bacteria, whereas the CF mucus is not.[6,15] Penetrability can be assessed by applying fluorescent beads with sizes corresponding to small bacteria (in this case, 2 μm). The beads were placed on top of the mucus formed on explants and were allowed to sediment for 60 min. Confocal z-stacks were acquired to evaluate mucus penetrability. In WT mice, the fluorescent beads (green) penetrated the mucus and sedimented down between the villi (tissue stained blue, Fig. 4a). In the CF mice, the beads were trapped at the surface of the mucus (Fig. 4b,c). In some CF tissues, the beads penetrated to the villus tips without penetrating the mucus between the villi; but in others, mucus separated the beads from the villi tips (Fig. 4c–e). When the CF mucus was incubated with 2% NaCl for 30–60 min, there was no increase in beads penetration. Instead, the increased mucus thickness (Fig. 3c) separated the beads and villi tips further (Fig. 4c,d). In some locations on two explants, the mucus was penetrable (Fig. 4f), a profile that is occasionally observed in this non-uniform system. Despite this variability, the mean separation of beads from villi tips was larger in CF ileum treated with HS (2% NaCl, 165 $\pm$ 37 μm) compared with untreated CF ileum (0.9% NaCl, 43 $\pm$ 14 μm), as shown in Fig. 5.

DISCUSSION

Although the basis for CF is a dysfunctional CFTR channel, the relationship of this defect to actual CF disease manifestations has been controversial, and there is still no uniform understanding of the reason for chronic lung infections. There are two major and partly opposing models for the consequences of CFTR dysfunction, one suggesting that the antibacterial peptides and proteins are less efficient, and the other that the pericilliary liquid is

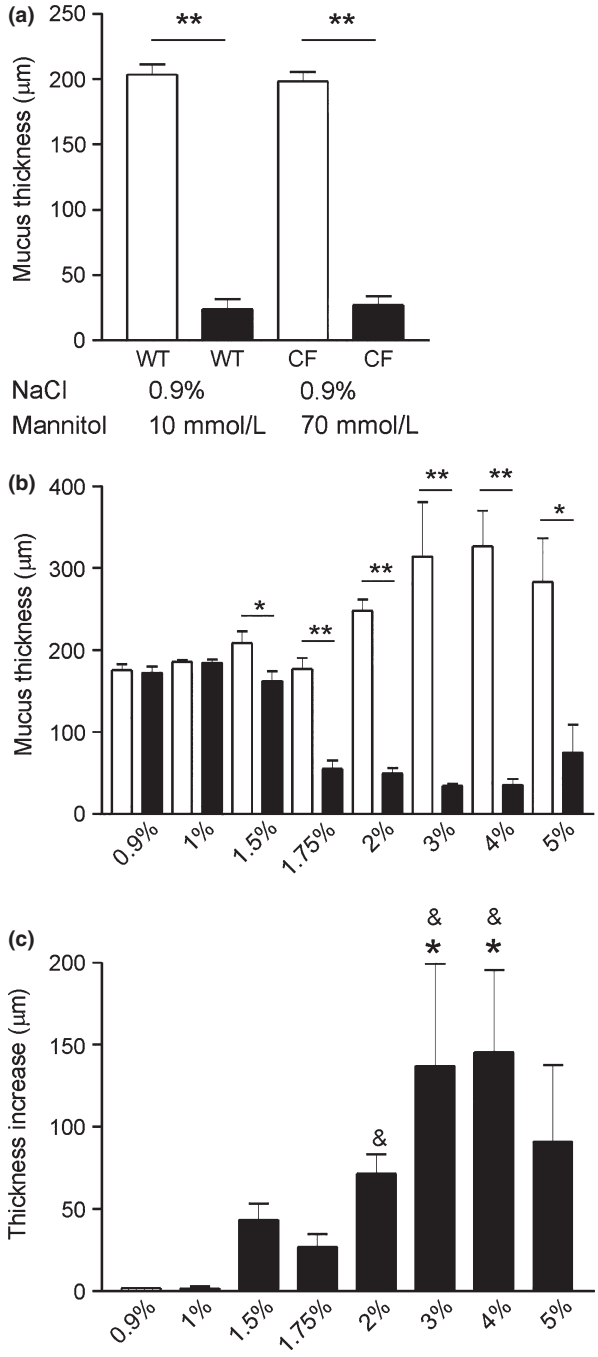

Fig. 3 Mucus was easily aspirated after incubation with hypertonic saline. (a) Mucus thickness was measured immediately before aspiration (white bars) and again after aspiration from normal homozygous wild-type (WT) small intestinal explants (black bars; $^{**}P = 0.008$, $n = 5$), and with 70 mmol/L mannitol before aspiration (white bars) and after aspiration from cystic fibrosis (CF) small intestinal explants (black bars; $^{**}P = 0.008$, $n = 5$). (b) Mucus thickness was measured immediately before aspiration and again after aspiration from CF explants. Mucus thickness after aspiration was significantly different from before aspiration in CF explants with 1.5% NaCl ($^{*}P = 0.03$), 1.75–4% NaCl ($^{**}P = 0.008$), and 5% NaCl ($^{*}P = 0.02$; $n = 5$). Note that 1.75% NaCl did not increase mucus thickness, but did release mucus from its epithelial attachment. (c) Mucus thickness increased over 1 h on CF ileal explants ($n = 5$) mounted in the chamber and incubated with apical solutions containing increasing concentrations of NaCl ($^{*}P < 0.05$ compared with 0.9% NaCl and $^{\&}P < 0.05$ compared with 1% NaCl).

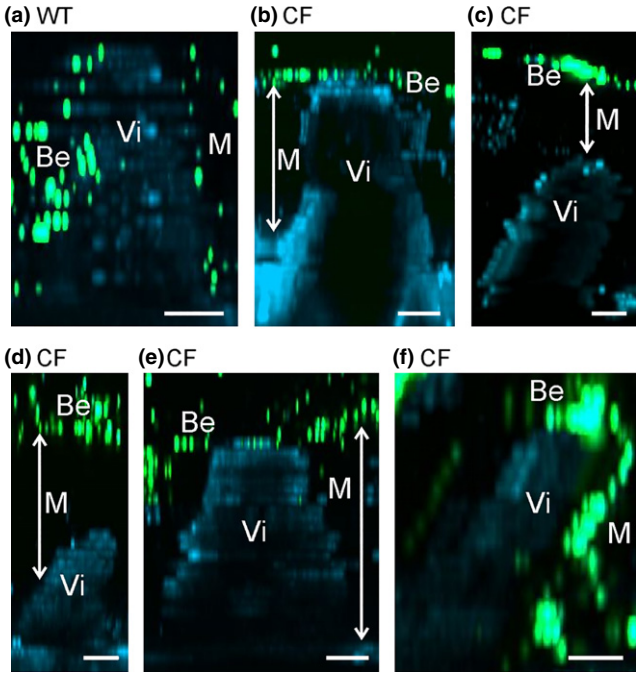

Fig. 4 Mucus was impenetrable to beads in the cystic fibrosis (CF) mouse ileum, also after incubation with 2% NaCl. (a) Ileal mucus formed on normal homozygous wild-type (WT) mouse explant was penetrable to 2-μm beads (green). Tissue, blue. (b) Ileal mucus formed on CF mouse explant was not penetrable to 2-μm beads, and the mucus only penetrated to the tips of the villi (n = 3). (c) In one animal, the separation between epithelium and beads was even larger, causing a separation between beads and villi tips. (a–c) The apical solution contained 0.9% NaCl. (d) After incubation with 2% NaCl in the apical solution for 60 min, the CF mouse ileal mucus was still impenetrable to beads (n = 6). (e) In some locations on the CF mouse ileal explants, the separation was similar to untreated CF mouse ileum. (f) Two of the explants were penetrable in some locations after incubation with 2% NaCl in the apical solution. Tissues are blue and beads (Be) are green. Bar, 50 μm. M, mucus, two-sided arrow denotes mucus thickness; Vi, villus.

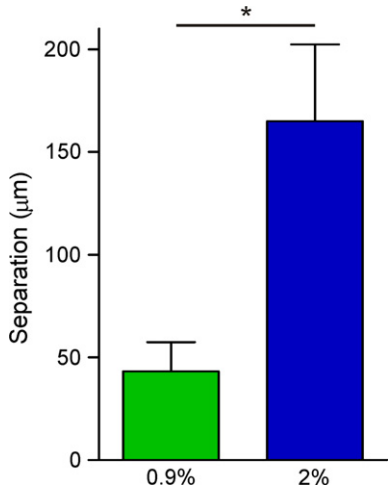

Fig. 5 The distance between beads and villi tips was larger in cystic fibrosis (CF) ileum treated with 2% compared with 0.9% NaCl. Mean separation of beads from the ileal villi tips in all the locations in CF mice (n = 3, green bar) and CF mice treated apically with 2% NaCl for 30–60 min (n = 6, blue bar). The separation was greater after incubation with hypertonic saline, *P = 0.001.

decreased, causing mucus to be trapped in the cilia and entangled in the glycocalyx.[18,19] The latter model was initially suggested to be caused by hyperabsorption of sodium, but is more likely a result of decreased chloride and water efflux.[19,20] More recently, the gel-on-brush model was proposed, indicating an electron-dense meshwork of transmembrane mucins between the cilia, resulting in a double-layered system. Also, this model suggests that the stagnant mucus characteristic of CF and COPD is a result of dehydration of mucus.[21,22] Irrespective of aetiology, the lungs of CF patients show poor bacterial clearance through the muco-ciliary system. Recently, it was suggested that the mucus strands formed from submucosal glands are attached in $Cftr^{-/-}$ but not $Cftr^{+/+}$ piglets. This is extremely interesting, because it suggests that there is a mucus phenotype before there is any sign of bacterial infection or inflammation in the lungs.[23] Because the lungs are the main site of the severe and life-shortening CF problems, less emphasis has been put on other organs. Among other hallmarks of CF are the salty sweat and plugged tubes of secretory organs, such as the pancreas, seminal ducts and biliary tree. Less attention has also been directed to the small intestine despite the high frequency of meconium ileus and distal obstruction syndrome. We have focused on this organ because the movement of mucus is slower and the main structural component is the MUC2 mucin, and because mouse CF models, both the full knock-out and ΔF508-Cftr, have an intestinal pathology similar to humans.[13] Our previous studies have shown that, in contrast to WT, the CF small intestinal mucus is attached to the epithelium, and that this a result of diminished unfolding of the intestinal MUC2 mucin. This was shown to be caused by insufficient concentrations of HCO_3^- during mucin secretion.[6] Hence, the important ion in this context is not chloride, but bicarbonate. We have also shown that selective CFTR inhibitors only cause 70% inhibition of the forskolin response in this set up, and this level of inhibition is not sufficient to cause induction of the CF phenotype (i.e. attached mucus) in the ileal explants. However, when the serosal solution was devoid of bicarbonate, the WT phenotype of detached mucus could be reverted to a CF-like phenotype with attached mucus. Thus, we conclude that a lack of bicarbonate causes the intestinal CF mucus pathology.[6] It is reasonable to consider that attached mucus might also be a feature of CF mucus in the airways, and therefore observations regarding the ileal mucus phenotype could be relevant to the lung.

Although the underlying mechanisms of CF lung problems are not fully understood, the use of inhaled HS has been a clinically successful treatment. The use of HS was first applied to CF in Australia, and later spread to the rest of the world after several promising clinical studies.[9,10] The use of HS solution (5–7%) is now accepted as a cornerstone of CF treatment in most countries.[9] The mechanism behind its effect has been suggested to be improved function of endogenous antibacterial components as a result of increased salinity of the periciliary layer (PCL) along with increased PCL depth caused by increased osmolality. A decreased PCL height in CF has been observed in human bronchial epithelial cultures obtained from CF patients in comparison with controls. It was also noticed that the mucus was intermingled and trapped within the cilia.[19,21] On normal human bronchial epithelial cultures, the mucus is transported in a circular motion (like

a 'hurricane'), whereas on cell cultures from CF patients, the mucus is immobile. When the PCL is too low, the mucus does not float on top of the cilia, the cilia cannot beat, and the mucus is trapped and immobile.[19] This model of trapped mucus is attractive as an explanation for the CF lung phenotype, because it suggests why clearance of bacteria from the lungs is poor.

Using methods developed for studies of the small intestinal mucus system, we have now shown that incubation of CF mucus with HS, from 1.75% to 5% NaCl, for 1 h on already formed CF intestinal mucus, released it from its attachment to the epithelium. The mucus also increased in thickness at the higher NaCl concentrations, but this was not the case at 1.75% NaCl, the lowest concentration when the mucus became easily removable. Thus, the thickness increase is not strictly coupled to release from attachment. It was also noticed that the increased mucus thickness did not cause the mucus to become penetrable to bacterial sized beads, showing that the mucus gel network is not affected by HS at these lower concentrations. Indeed, the beads were still trapped in the mucus, further indicating that some aspects of mucus properties were not affected by HS. Together, these results suggest that the thickness increase is not coupled to the release of mucus from its attachment, and that the mucus release and detachment are separate processes. We cannot fully explain the mechanism behind the detachment of mucus by HS, but it is not a result of increased bicarbonate concentration, as it is highly unlikely that HS stimulates carbonic anhydrase to cause formation of bicarbonate to the extent that the apical concentration reaches 100 mmol/L. Furthermore, carbonic anhydrase is the only conceivable source of bicarbonate, as the serosal concentration of bicarbonate is 23 mmol/L in this experimental set up. It is also highly unlikely that the tissue itself could concentrate bicarbonate to 100 mmol/L by selective transport. Furthermore, bicarbonate induces penetrable mucus in the CF mouse ileum, whereas HS does not affect CF mucus impenetrability.[6] We conclude that treatment of CF small intestinal mucus with HS causes detachment of mucus from the epithelium by a process unrelated to bicarbonate. Bacteria are trapped in the mucus and cleared when mucus is removed.

Although our observations cannot be directly transferred to the respiratory system, we can still suggest similarities. If mucus could be more easily removed from the lungs, this should be beneficial for the CF patient, as it would avoid mucus stagnation and bacterial overgrowth. From the present results, it is apparent that the increase in NaCl concentration does not have to be dramatic to obtain a significant effect on the mucus, as increasing the NaCl concentration from 0.9% in the normal Krebs' buffer to 1.75% was sufficient to detach the mucus. Interestingly, assuming an airway liquid volume of 2 mL and a 15% distribution (GastroPlus 8 software, Simulations Plus, Lancaster, CA) shows that inhaling a 7% NaCl solution could result in local NaCl concentrations in the range that caused observable mucus detachment in our system.

The remaining question is the mechanism for HS in the ileum. The HS-induced CF mucus detachment was mimicked by apical incubation of the ileal explants with a 70-mmol/L mannitol solution, showing that osmolarity rather than ionic strength is essential for mucus detachment. This is another parallel to the airways, where inhaled mannitol has been shown to improve lung function.[24] The thickness increase was moderate, and did not occur at the same concentrations as the detachment, making it less likely that the mechanism is linked to drawing water or bicarbonate out of the epithelium. We have previously shown that the MUC2 mucin is packed in the goblet cell granulae as a result of interactions in the N-terminal part of MUC2 caused by high Ca^{2+}/low pH.[6,14] With intestinal mucus, unfolding requires an increase in the pH and removal of Ca^{2+} ions, both of which are mediated by bicarbonate ions. The increased Na^{+} concentration will compete with the Ca^{2+}-binding to the mucin N-terminus and as removal of Ca^{2+} is a prerequisite for expansion, Na^{+} ions can thus promote mucus unfolding. Unfolding of the MUC2 N-terminus exposes hidden proteolytic cleavage sites that are necessary for release of the mucin from its attachment. However, for the respiratory tract, it is not yet known if the mucins are anchored in a similar way as in the intestine, and if release from the attachment also requires enzymatic activities.

The techniques used to evaluate mucus thickness and properties in the gastrointestinal tract have now been proven useful for the study of CF intestinal mucus.[6,16] We have previously shown that a bicarbonate concentration of 100 mmol/L can release attached mucus, and we now show that a hypertonic solution of 1.75% NaCl can have the same effect. The present study findings suggest that these Ussing-chamber techniques can be valuable for research and evaluation of therapeutic strategies for treating the CF mucus phenotypes and its consequences.

METHODS

Animals

Male and female (age 8–16 weeks) homozygous ΔF508-Cftr mice on C57BL/6 background (backcrossed for 13 generations) were obtained from the Erasmus MC animal facility, and bred as heterozygotes and with C57BL/6 mice (Taconic) at Gothenburg University as described.[13,25,26] Mice were housed in individually ventilated cages under controlled temperature (21–22°C), humidity and 12-h light/dark cycle under specific pathogen-free conditions. All mice were killed by cervical dislocation under isoflurane anaesthesia. Ethical approval was granted by the Laboratory Animal Ethics Committee, University of Gothenburg, and experimental animal care was in accordance with their guidelines.

Explants

Intestinal explants were prepared and mounted as described previously.[16] Briefly, to visualize the otherwise transparent mucus surface through a stereomicroscope at ×40 magnification (Leica MZ125, Wetzlar, Germany), activated charcoal particles in Krebs' mannitol buffer were added and allowed to sediment on top of the mucus. Videos were recorded using a Leica IC D 3.3 megapixel camera mounted to the microscope, and mucus was aspirated with a Pipetman P200 (Gilson, Middleton, WI, USA) set to 150 μL and a yellow tip. Tissue viability was monitored by PD (Ref201; Radiometer, Copenhagen, Denmark) connected to the chamber by agar bridges (4% agar, 0.9% NaCl).

Mucus thickness measurements

Mucus thickness was measured as described, from the mucus surface to the villus tips every 20 min for 1 h, the last time-point

being denoted 'Pre-aspiration' in the figures.[15] To evaluate mucus properties, the whole apical volume was aspired using a plastic Pasteur pipette (PP-101, outer tip diameter 0.9 mm, inner tip diameter 0.7 mm, maximum volume 800 μL; Cellprojects, Sutton Valence, UK) by placing the pipette opening at the edge of the circular opening, taking care not to touch the mounted tissue and kept in place while releasing the bulb during approximately 3 s, thus removing all removable liquid. The remaining mucus thickness (denoted 'Post-aspiration' in the figures) was measured after refilling the apical chamber with 150 μL Krebs' mannitol to ensure any mucus material is supported and does not collapse, and adding new charcoal particles. Finally, the villus height was measured from the epithelium between the villi to the villi tips. Total mucus thickness is presented as the sum of villus height and mucus on top of the villus.

Mucus penetrability

For penetrability measurements, ileal explants from CF and WT mice were mounted in a horizontal imaging chamber (RC-50; Warner Instruments, Hamden, CT, USA) with a 1.8-mm^2 opening and 1.5 mL Krebs' mannitol buffer (in mmol/L: 116 NaCl, 1.3 $CaCl_2$, 3.6 KCl, 1.4 KH_2PO_4, 23 $NaHCO_3$, 1.2 $MgSO_4$ and 10 D-mannitol, pH 7.4) was added to the apical chamber. The serosal side was constantly perfused (at a rate of 5 mL/h) with Krebs' glucose solution (10 mmol/L glucose instead of mannitol) containing CellTrace BODIPY TR Methyl Ester (10 μmol/L; Invitrogen, Carlsbad, CA, USA) to visualize the tissue. As for mucus measurements, the chamber was heated to 37°C during a period of 10 min, using a temperature controller (Harvard Apparatus, Holliston, MA, USA), and thereafter kept at a constant temperature. After temperature equilibration, the apical buffer was removed, and a 5 μL suspension of 2 μm (green) fluorescent beads (FluoSpheres; Invitrogen) in Krebs' mannitol was added to the apical surface. The beads were allowed to settle in the mucus for 5 min before new Krebs' mannitol buffer was added to the apical chamber, and then the beads were left to sediment through the mucus for 30–60 min. Confocal Z-stacks (optical section 2.8 μm, interval 10 μm) were taken to analyse the distribution of the different beads throughout the mucus, using an upright LSM 700 Axio Examiner 2.1 confocal imaging system with a Plan-Apochromat ×20/1.0DIC water objective (Carl Zeiss, Oberkochen, Germany). IMARIS (Bitplane, Zurich, Switzerland) software was used to process images. Representative Z-stacks are shown to illustrate bead penetrability.

Immunofluorescent stainings

Whole tissues from CF and WT mice were fixed in Carnoy's fixative (60% dry methanol, 30% chloroform and 10% glacial acetic acid), embedded in paraffin and cut in 4-μm thick sections. The sections were dewaxed using Xylene substitute (Sigma, St. Louis, MO, USA) and hydrated. Antigen retrieval was carried out by microwave heating in 0.01 mol/L citric buffer pH 6, and the sections were stained with custom-made anti-MUC2C3 antiserum (1 : 500) and goat anti-rabbit Alexa 488 secondary antibody (Invitrogen).[27] DNA was stained by TO-PRO-3 Iodide (1 μmol/L, 642/661; Invitrogen). Images were acquired using a fluorescence microscope, Eclipse E1000 with a Plan-Fluor 40×/0.75 DIC objective (Nikon, Amstelveen, the Netherlands). The pictures were processed uniformly using ADOBE PHOTOSHOP (Adobe, San Jose, CA, USA).

Data are presented as mean ± standard error of the mean (SEM) for n animals. The Mann–Whitney test was used to test differences between two groups and the Kruskal–Wallis with Dunn's multiple comparisons test was used to test differences between multiple groups. Statistical significance was accepted when $P < 0.05$.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Movie S1. Aspiration of easily removable normal homozygous wild-type mucus from an ileal explant mounted in the Ussing-type chamber.

Movie S2. Attempt to aspirate the attached cystic fibrosis mucus from an ileal explant mounted in the Ussing-type chamber.

Movie S3. Aspiration of cystic fibrosis mucus after treatment with 2% NaCl for 40 min from an ileal explant mounted in the Ussing-type chamber.