Pathological Hyaluronan Matrices in Cystic Fibrosis Airways and Secretions

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Abstract

Hyaluronan (HA) has been used in treatment of cystic fibrosis (CF) via a nebulizer and has demonstrated success in clinical outcomes. HA is an important glycosaminoglycan that is cross-linked by heavy chains (HCs) from inter- α -inhibitor during inflammation. HC cross-linked HA (HC-HA) becomes significantly more adhesive for leukocytes than non-cross-linked HA, which can enhance inflammation. Our studies tested the hypothesis that HC-HA is present in CF airways and that altered ratios of HC-HA to its degradation into relatively lower molecular weight HA contribute to the pathophysiology of chronic inflammation in CF. We evaluated the distribution, levels, and size of HC-HA within CF, healthy, and diseased control lung, bronchus, and sputum tissues by histological and biochemical approaches. HC-HA was significantly elevated in CF, with deposits around the pulmonary vasculature, airway submucosa, and in the stroma of the submucosal glands. The increased infiltration of leukocyte populations correlated with the distribution of HC-HA matrices in the airways. Elevated lung tissue HC-HA correlated with decreased HA levels in CF mucus and sputum compared with

controls, suggesting that aberrant degradation and cross-linking of HA in lung tissue is a unique feature of CF. The accumulation and degradation of proinflammatory HC-HA in CF lung tissue suggests that aberrant HA catabolism and cross-linking may contribute to chronic inflammation in airway tissues and affect mucus viscosity in CF airways.

Keywords: hyaluronan; TNF-stimulated gene-6; cystic fibrosis; sputum; inflammation

Clinical Relevance

Our studies are the first to demonstrate the accumulation of heavy chain-hyaluronan (HA) in cystic fibrosis (CF) lung tissue, which, in the unique CF pulmonary milieu, ultimately degrades into lower molecular weight (MW) products. The enhanced destruction of high-MW antiinflammatory HA contributes to the chronic pulmonary inflammation observed in CF.

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No drugs were used in this study. Manufacturers of reagents used in this study are listed within the MATERIALS AND METHODS where indicated.

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Hyaluronan (HA) belongs to a class of polysaccharides known as glycosaminoglycans (GAGs) (1, 2). Other members of this class include chondroitin sulfate (CS), heparan sulfate, and keratan sulfate. HA is a linear polysaccharide composed of a repeated disaccharide unit of glucuronic acid and an amine containing monosaccharide (*N*-acetyl-glucosamine). HA differs from the other GAGs in several ways, including: (1) it is synthesized at the cell surface by HA synthases, which extrude it into the extracellular space without a covalently attached core protein (2); (2) HA (>2,500 kD) is much larger than other GAGs (20–100 kD); and (3) HA is not sulfated.

In a healthy lung, HA is primarily present around the smooth muscle cells within the vascular wall, in the submucosa underlying the airway epithelium, and can be quantified in the airway surface liquid (3). During inflammation, HA accumulates in these sites, seemingly to amplify leukocyte recruitment. Inflammation can also involve the alteration of HA function and bioactivity by the covalent substitution of HA in the extracellular matrix with heavy chains (HCs) from the proteoglycan inter- α -inhibitor (I α I) (4–6) and degradation of HA into smaller bioactive fragments (7, 8).

HC modification of HA is accomplished by the enzyme TNF-stimulated gene 6 (TSG-6) and I α I, which are regulated at sites of inflammation. The substrate of TSG-6, I α I, is delivered to sites of inflammation in serum exudates via dilated vasculature, where it is spatially and temporally coordinated with HA deposits and the secretion of TSG-6. When HA, I α I, and TSG-6 come together, TSG-6 covalently transfers the “heavy chains” of I α I to hydroxyl groups on the sixth carbon of HA’s *N*-acetyl-glucosamine groups. This process results in HC cross-linked HA (HC-HA), ultimately transforming its structure and augmenting the adhesion of leukocytes to HA matrices (9–11). This dynamic modification of HA and its contribution to inflammation is modeled in Figure 1.

In chronic inflammation, HA remodeling continuously occurs, producing an abundance of HC-HA matrix that is fragmented by invading inflammatory cells. These HA fragments are then released into the extracellular fluid space, which can participate in response to pathogens or airway sensitivity (3). Such HA fragments can be recognized as damage-associated

Figure 1. Hyaluronan (HA) “cross-linking” with heavy chains (HCs). Normally, HA is present in airway tissue and secretions as a large glycosaminoglycan void of protein substitutions. During inflammation, a large portion of HA is enzymatically “cross-linked” (Step 1) by the enzyme TNF-stimulated gene 6 (TSG-6) (orange) via the HCs (green) of the cross-link donor inter- α -inhibitor (I α I). HC cross-linking of HA matrices significantly increases the avidity of inflammatory cells (purple; Step 2), which ultimately degrade HA matrices into smaller bioactive fragments, prolonging inflammation and compromising mucociliary clearance of pathogens (Step 3).

molecular patterns by several pathogen-recognition receptors, including Toll-like receptor 4 (TRL4), myeloid differentiation primary response gene 88 (MYD88), and Toll-IL-1R domain-containing adapter protein (TIRAP) (12). Whether interrelated or not, HA also contributes to downstream signaling pathways, which regulate airway hyperreactivity and the production of proinflammatory cytokines, which exacerbate reactive airway disease observed in asthma (12).

The role of HA in the pathophysiology of cystic fibrosis (CF) lung disease has not been determined. Previous reports have demonstrated that nebulized HA has the capacity to improve clinical outcomes in subjects with CF (13–17), as well as reverse some aspects of pathology in murine models of CF infection and inflammation (18). In these studies, we show increased levels of HC-HA along with the skew of HA toward a relatively lower molecular weight (MW), suggesting aberrant HA processing in the unique CF lung milieu.

Materials and Methods

CF Lung Tissue and Sputum

All healthy and diseased patient lung tissue and sputum were obtained with Cleveland Clinic (Cleveland, OH) Internal Review Board approval. Histological paraffin section staining was performed on CF lungs obtained at the time of lung transplantation and from rare, large surgical biopsies of healthy control subjects. Tissues processed for Western blotting were obtained from non-CF lungs rejected for lung transplant. Non-CF control sputum was obtained as discarded specimens from patients with chronic obstructive pulmonary disease, lung cancer, after lung transplant, asthma,

pneumonia, and/or croup/bronchiolitis. CF sputum was obtained from the CF center at Rainbow Babies Hospital (Cleveland, OH; Δ F508 phenotype). Sputum that was infected with *Burkholderia cepacia*, tuberculosis, or nontuberculosis mycobacteria was excluded. The protocols described in the following sections were performed, and can be found in detail at <http://pegnac.sdsc.edu/cleveland-clinic/protocols/>.

Histochemistry and Fluorescent Microscopy

This method was previously described (6). Paraffin sections from CF and normal human lung/bronchus tissue were labeled with a biotinylated HA binding protein (HABP; 5 μ g/ml; 385911; EMD/Millipore, Gibbstown, NJ). I α I was labeled with a rabbit polyclonal antibody (1:100; A0301; Dako, Glostrup, Denmark). CD45 was labeled with a mouse monoclonal antibody (1:100; M070; Dako). Imaging was done by standard fluorescent microscopy and pseudocolored using ImageJ (National Institutes of Health, Bethesda, MD). Immunohistochemical staining was performed with HABP using the VectaStain Elite ABC Kit (Vector Laboratories, Burlingame, CA).

HA-HC Analysis by Western Blot

This method was previously described (6, 19). Human CF and control lung/bronchus tissue were processed and blotted using Bio-Rad nitrocellulose and Trans-Blot Turbo System (Biorad, Hercules, CA), probed with a rabbit polyclonal antibody against I α I (1:8,000 dilution), and the secondary was anti-rabbit 800CW (1:15,000; 926-32211; Li-Cor, Lincoln, NE). Blots were imaged on an Odyssey Infrared CLx Imaging System (Li-Cor).

Fluorophore-Assisted Carbohydrate Electrophoresis of Lung and Bronchus Tissue, and Sputum

This method has been described previously (20, 21).

HA Size Analysis by Agarose Gel Electrophoresis

This method was previously described (21). HA profile line scans from two non-CF and two CF specimens run on the agarose gels each generated 720 discrete data points. These datasets were transformed into 35 discrete data points for each HA profile by integrating every 20 consecutive original data points. This allowed for determining group-averaged means, SDs, and statistical analysis.

HA Quantification from Sputum Samples

The sputum wet weight and dry weight (after dehydration on a centrifugal vacuum concentrator), were measured. This calculation of the concentration of dry/wet weight permitted the analysis of a specific amount of sputum normalized (4.4 mg) by its dry weight. For fluorophore-assisted carbohydrate electrophoresis (FACE) and HA size analysis, sputum was digested exhaustively with proteinase K.

Hyaluronidase Assay

Sputum was normalized by dry weight by transferring a known volume of sputum (average, 129.6 mg wet weight), lyophilizing it (average, 6.8 mg dry weight), and adding PBS so the final concentration of sputum was 32.2 μg dry weight/ μl PBS.

Statistical Analysis

Quantitative data were summarized as means ($\pm$ SD) unless otherwise noted. *F* tests and *t* tests were used at the confidence interval of 95%, designating statistical significance with a *P* value of 0.05 or less as significant.

Results

Accumulation of HA Matrices in CF Lung and Bronchus

The distribution and relative levels of HA by immunohistochemistry of CF and control lung and bronchus tissue were investigated using a biotinylated HABP. In Figures 2A and 2B, the HA distribution in the control lung tissue was primarily

in the underlying connective tissue of the vasculature and airways, with less localizing to the alveoli wall. Furthermore, the HA was present in the surrounding connective tissue of the healthy control bronchus (Figures 2C and 2D). As seen in Figures 2E and 2F, HA distribution was similar in CF lung tissue and was predominant in the fibrotic and inflamed regions surrounding the airway epithelium and connective tissue, interestingly without significant detection in the CF mucus. In CF bronchus tissue (Figures 2G and 2H), HA distribution was broad and included the connective tissue surrounding the submucosal glands. HA distribution in the CF submucosal gland (Figure 2H) demonstrates HA that is colocalized with inflammatory cells around the gland structures.

Characterization of HA's HC Modification in the CF Bronchus

Although the distribution of HA within CF lung and bronchus tissue was similar, our data suggest that the HA may be more prominent in specific regions of the CF specimens than in healthy control tissue (see Figure 2). The prominence of HA in specific regions of the CF specimens may be due to a greater amount of HC modifications of HA that permits continued accumulations of HC-HA as opposed to unmodified HA, which normally is resorbed and replaced by cells in a homeostatic mass balance. To determine if this difference in prominence is due to HC modifications, we used antibodies to colocalize HA and HC-HA in both the control and CF bronchus. Figure 3 visually demonstrates that colocalization of HA (*green fluorescence*) and the HCs of I α I (*red fluorescence*) was observed in the matrix surrounding CF submucosal glands.

HA is distributed throughout the glandular connective tissue in the healthy control tissue (Figure 3A), with I α I matrix absent (Figure 3B). In the CF submucosal glands, there is evident colocalization of HA with I α I in the glandular matrix (Figures 3D–3F), suggesting a more regional distribution of the HA/I α I complex within the CF bronchus. It should also be noted that inflammatory cells are abundant within the interglandular spaces in the CF specimens, and that HA and I α I colocalize in this region (suggesting the presence of

HC-HA). These studies suggest that the modification of HA with HCs is not uniform throughout the tissue in CF, which may be related to specific milieu differences associated with either deficient CF transmembrane conductance regulator (CFTR), inflammation, or concurrent infection.

Hyaluronidase Extraction of HCs from CF Lungs and Bronchus

Although the colocalization of HA with I α I (Figures 3D–3F) provides evidence that HA may be modified with HCs from I α I, it is possible that I α I independently colocalized with HA in the absence of covalent bonding to HC. This might occur if serum exudate, containing I α I, entered the inflamed tissue in the absence of TSG-6 activity, thus bathing HA matrices with I α I, but not actually involving the covalent transfer of HCs to HA. To test this hypothesis, we treated minced CF and control tissue with and without *Streptomyces* hyaluronidase, which digests the HA of HC-HA complexes into hexa- and tetrasaccharides, thereby releasing HCs from high-MW HC-HA complexes and resulting in an 83-kD band by acrylamide gel electrophoresis. In the absence of hyaluronidase, the HC-HA complex is too large to enter into the gel. Tissue from CF bronchus (Figure 3J, lanes 3 and 4) and lung tissue (Figure 3J, lanes 7 and 8) treated with hyaluronidase resulted in degradation of the HC-HA substrate, demonstrating the ample presence of the 83-kD HC band in the +hyaluronidase treatment group. Treatment of control lung tissue with hyaluronidase yielded a weaker 83-kD HC band than that for the CF tissue (Figure 3J, lanes 1, 2, 5, and 6), as shown by quantification of the HC bands (Figure 3K). The quantification values shown in Figure 3K represent two sets of non-CF and CF pairs, each run on separate gels. In each case, the HC band in CF lung tissue yielded a band 1.5- to 2.7-times stronger than that of non-CF controls. An interesting distinction between the two control samples was noted regarding the higher MW bands attributed to I α I and pre-I α I that are normally found in serum. The non-CF control specimens were obtained from biopsies of tissue deemed unsuitable for lung transplantation, and many biopsies likely contained serum from contaminating blood pools in the tissue (Figure 3J, lanes 5 and 6). The control

Figure 2. Accumulation of HA matrices in cystic fibrosis (CF) lung and bronchus tissue. HA staining (brown) was performed on paraffin lung (A–B and E–F) and bronchus (C–D and G–I) sections from patients with CF (E–I) and healthy control lungs (A–D). A negative control (I) shows a section probed with all reagents except the primary antibody. The images shown are representative of other replicates from different patients ($n = 6$ for CF and $n = 9$ for control lungs/bronchi). Nuclei were counterstained with hematoxylin. Scale bars shown are 2 mm (A, C, E, and G), 500 μm (B and F), and 200 μm (D and H). B and F (and D and H) are higher magnification images of the dashed boxed regions outlined in A and E (and C and G). Mucus (*) is shown in F and connective tissue (arrows) is annotated in B, D, F, and H. Alveoli (A) is annotated in B and F, and submucosal glands (SG) in D and H. Inflammatory cells are noted by arrowheads in H.

lung tissue shown in Figure 3J (lanes 1 and 2) was nearly devoid of blood serum $\text{I}\alpha\text{I}$ and pre- $\text{I}\alpha\text{I}$. These specimens are not the rare biopsies of healthy lung and bronchus tissue shown in Figures 2 and 3A–3I.

Leukocytes are Concentrated along HC-Modified HA Matrices in CF Airways

Leukocyte recruitment into the lung was associated with HA and $\text{I}\alpha\text{I}$. As shown in Figure 4, inflammatory cell accumulation was observed within the airway submucosa (defined by CD45-positive staining cells in magenta), directly basal to the airway epithelium, but largely apical to the underlying smooth muscle cell region. The presence of CD45 cells in this region of

the airway corresponds to the generalized location of inflammation. The majority of the HA (green) and $\text{I}\alpha\text{I}$ (yellow) staining was found beneath the tissue region having CD45 cell accumulation, but was largely absent in the overlying airway epithelium. Nevertheless, some leukocytes were found embedded within the apical-most layers of the HC-HA matrices of the CF airway submucosa.

HA and CS Levels in CF Lung and Bronchus Tissue

Total HA and CS concentrations were quantified by FACE analysis of both control and CF lung/bronchus tissue (Figures 5A–5C). CS data were calculated by quantifying the abundance of its two major disaccharides (sulfated at C4 and C6 of

N-acetyl-galactosamine (GalNAc); unsulfated chondroitin was barely at detectable levels). Using this technology, HA and CS levels did not differ significantly between CF and control lung/bronchus tissue. The implication is that, although total HA levels do not appear to be substantially different between control and subjects with CF, the modification of the HA with HCs appears to be elevated in CF.

Decreased MW HA in CF Lung Tissue

The bioactivity of HA is reported to be size dependent (7, 22), and possibly related to the role of HA in lung inflammation. Both control and CF lung and bronchus tissue were measured for size differences using agarose sizing gels (Figures 5D–5F). Control lung tissue (Figure 5D, lanes 2 and 3)

Figure 3. Characterization of HC-HA modification in CF bronchus. Fluorescence microscopy was performed on paraffin lung/bronchus sections from patients with healthy control lungs (A–C) and CF (D–F). These sections were probed with HA binding protein (HABP) for HA (green; A and D) and α 1 for the presence of HC (B and E). Overlays of the images are shown in C and F. 4',6-diamidino-2-phenylindole–stained nuclei are shown in blue. Negative controls, lacking primary antibody, are shown in G–I. These images are representative of other replicates from different patients ($n = 2$ for CF and $n = 6$ for control lungs). Submucosal glands (SGs), and bronchial cartilage (asterisk) are annotated in C, F, and I. Scale bar shown in I is 200 μ m. J shows as *Streptomyces*

Figure 4. Leukocytes localize near HC-HA matrices in CF airways. A paraffin section of CF bronchial tissue was probed with (A) the common leukocyte antigen CD45 (magenta), (B) HABP (green), and (E) Ial (yellow) (a marker for the HC-HA). Panels A and D (CD45, magenta) are displayed twice in order to show the overlay of CD45 and HA (C), and the overlay of CD45 and Ial (F). An overlay of CD45, HA, and Ial is shown in panel H. A serial section was probed with only secondary antibodies as a negative control (G). Arrows show the apparent boundary of the epithelium and the airway submucosa (E). Similar staining was observed in three different patients with CF in both bronchi and lung tissue (not shown).

had a higher MW distribution than CF lung tissue (Figure 5D, lanes 4 and 5). Treatment of these samples with *Streptomyces* hyaluronidase specific for HA (Figure 5D, lanes 7–10) served as a negative control for the identification of HA among the other GAGs, which typically appear as blue (chondroitin), purple (CS), and orange/brown (heparan sulfate) smears when stained by this method (21, 23). Figure 5E shows another CF lung replicate (lanes 12 and 15) as well as the MW distribution of HA in a CF bronchus (lanes 13 and 16). A line plot of the average data shown in Figure 5D is presented in Figure 5F. Using the HA standards, a correlation plot between relative mobility and log value of the HA standard MWs was completed, allowing a linear regression analysis (see Figure E1 in the online supplement). Using this calibration plot, the MW for the peak

value of non-CF lung tissue was calculated to be 9,100 kD (blue arrowhead), whereas the MW of peak value of CF lung tissue was 3,900 kD (red arrowhead). This lower MW distribution of HA in CF lung tissue is evident in the rightward migration of the HA optical density. Noted also was a statistically significant ($P < 0.05$) twofold increase in HA chains of 130–2,500 kD in the CF as compared with the non-CF specimens. The control tissue seen in Figures 5D–5F was similar to that presented in Figures 3J and 5A–5C.

Decreased HA in CF Sputum

The delivery of nebulized HA to CF airways has recently been reported to have a beneficial therapeutic effect (13–18). One hypothesis to explain this therapeutic effect is that HA levels are abnormally low in CF airway secretions and that the

delivery of nebulized HA may raise the mucus HA content back to normal levels. To test this hypothesis, we measured control and CF sputum HA and CS levels by FACE analysis (Figures 6A–6C). Sputum was normalized by the sputum dry weight (as described in the MATERIALS AND METHODS). Because sputum is not produced by healthy individuals in appreciable quantities, the non-CF sputum used in this study came from individuals with chronic obstructive pulmonary disease, lung cancer, or idiopathic pulmonary fibrosis (as described in the MATERIALS AND METHODS section). We found a statistically significant ($P < 0.0001$) decrease (65%) in the HA content of CF sputum compared with non-CF controls (Figure 6B). In contrast, CS levels were not different from controls (Figure 6C). It should also be noted that none of these patient samples was exposed to nebulized HA therapy.

HA Stability in CF Sputum

One hypothesis to explain the lower levels of HA in CF sputum is that any HA produced is degraded by endogenous hyaluronidases, cleaved by free radical damage or by some other mechanism(s). To evaluate the HA-degrading potential of CF sputum, a commercial source of purified HA (1,700 kD; Figures 6D and 6E) was added to CF and control sputum specimens to test for hyaluronidase activity. Positive controls (Figure 6D, wells A1 and B1) included the addition of *Streptomyces* hyaluronidase, whereas negative controls (Figure 6D, wells A2 and B2) were incubated in saline alone. Sputum volumes had been normalized by the sputum dry weight (as described in the MATERIALS AND METHODS). After an overnight incubation at 37°C (physiologic and isotonic conditions), only one CF sputum sample (Figure 6D, well B6) demonstrated any potential degradation of the HA. Overall, the stability of HA in CF and control sputum was comparable (Figure 6E), indicating that any endogenous HA degrading capacity was comparable between the diseased controls and CF specimens. Future studies will need to pursue sputum sources not associated with on-going pulmonary inflammation.

Figure 3. (Continued). hyaluronidase release of HCs from HC-HA matrix from CF lung and bronchus tissue CF lung/bronchi tissue treated with or without *Streptomyces* hyaluronidase. Samples were analyzed by Western blot, using an antibody that detects the HCs (green) of Ial (cartoon shown to the right of the blot). The red arrow points to the 83-kD bands (J). MW standards are shown in red. Samples from two different control lung patients are shown in lanes 1, 2 (#1), 5, and 6 (#2). Quantification values of the HC bands representing two sets of non-CF and CF lung tissue pairs (each pair run on separate gels) are shown in K.

Figure 5. HA and chondroitin sulfate (CS) characterization in CF lung and bronchus tissue. HA and CS levels in CF and control lung tissue were quantified by fluorophore-assisted carbohydrate electrophoresis (FACE). (A) The HA and CS levels from two control (lanes 2 and 3), three CF lungs (lanes 4, 5, and 7), and one CF bronchus. Mono- and disaccharide standards are shown in lanes 1 and 6; lane 9 portrays a 2-aminoacridone (AMAC) only (loading) control. (B and C) Mean $\pm$ SD values of HA and CS from lung and bronchus tissue, respectively. (B) Replicates from lung tissues from 10 control (non-CF) and 3 patients with CF. (C) The HA and CS contents from a single CF bronchus. The size distribution of HA in CF lung and bronchus tissue was analyzed by agarose gel electrophoresis. HA was stained by “Stains All” and appears as blue bands/smears in the upper half of the gels (D and E). “Select HA” (Hyalose) molecular weight (MW) standards are shown in lanes 1, 6, 11, and 14; control lung tissue in lanes 2, 3, 7, and 8; and CF lung tissue in lanes 4, 5, 9, 10, 12, and 15. CF bronchus tissue was loaded into lanes 13 and 16. Parallel samples predigested with *Streptomyces hyaluronidase* are shown in lanes 7–10, 15, and 16, whereas samples not digested are shown in lanes 2–5, 12, and 13. (F) The mean $\pm$ SD profiles of control lungs (lanes 2 and 3) and CF lungs (lanes 4 and 5) plotted against the HA MW standards. The MW for the peak values of non-CF samples (9,100 kD, blue arrowhead) versus that of CF samples (3,900 kD, red arrowhead) are also shown in F. Ctrl, control; Δ Di, unsaturated disaccharide; GAG, glycosaminoglycan; GalNAc, *N*-acetyl-galactosamine.

Discussion

In this article, we have presented an overview of the altered distribution and levels of HA in CF airways and sputum. The major conclusions from this study are that the HA present in CF tissue: (1) is covalently modified with the HCs from I α I; (2) has a lower MW distribution than in control tissues, and (3) has levels in CF sputum significantly lower than in control sputum.

The HC modification of HA is the only known covalent modification to naturally occur with HA (24, 25). Normally devoid of protein modifications of any kind when first

synthesized, the covalent transfer of HCs to HA in the extracellular space creates a novel ligand that functions as a binding site for leukocyte adhesion to HA matrices (9–11). The transfer of HCs to HA creates a macromolecular aggregate that can also include other hyaladherins (i.e., HABPs), such as versican and pentraxin-3 (26, 27). HC transfer is mediated by regional expression of the enzyme TSG-6, which is a novel transesterase that transfers the HCs from the C6 hydroxyl of an GalNAc residue of bikunin's CS chain in I α I to a C6 hydroxyl of *N*-acetyl-glucosamine residues on HA (24). We observed that

HA matrices in CF lung tissue are largely substituted with these HCs and that inflammatory cells were found aggregated along this matrix's apical surface. One of the known functions of this HC modification is to make HA “sticky” for leukocytes (9–11). Whether HC-HA is a ligand for a specific receptor on leukocytes has yet to be determined. Alternatively, the attachment of HCs to HA might simply organize HA matrices into a tertiary structure that engages one or more receptors on leukocyte surfaces. The leukocyte-adhesive HA “cable” structure may potentially result in an intertwining of

Figure 6. CF sputum levels of HA and CS. **A** shows a representative FACE gel from control (lanes 3–6) and subjects with CF (lanes 7–12). Sputum HA and CS levels were quantified by densitometry of the Δ DiHA and Δ DiOS/ Δ Di6S/ Δ Di4S bands, respectively. Additional CF replicates on a separate FACE gel (gel not shown) were compiled with the results from the gel in **A** and were incorporated into the graphs in **B** and **C** (control, $n = 4$; CF, $n = 10$; mean $\pm$ SD). Disaccharide standards are shown in lane 1, and a loading control (AMAC only) is shown in lane 14. The sample in lane 2 is from a control subject whose sputum was mostly saliva and was not included in the analysis. The sample in lane 13 was a tracheal aspirate (not sputum) from a control subject and was not included in the analysis. **(D and E)** The stability of HA covalently attached to eight-well strips after overnight incubation with CF sputum at 37°C. Sputum volumes had been normalized by the sputum dry weight. **(D)** Two eight-well strips incubated with control sputum (yellow circles; $n = 5$), CF sputum (red circles; $n = 7$), a negative control in which PBS was added instead of sputum (green circles), and a positive control in which the well was incubated with *Streptomyces* hyaluronidase instead of sputum (blue circles). The green color in each well represents the amount of HA remaining bound to the plate after the incubation. The optical density (OD) of these wells was quantified and presented as a bar graph in **E** (mean $\pm$ SD). Δ DiHA, 2-acetamido-2-deoxy-3-O-(β -D-glucopyranosyluronic acid)-D-glucose; Δ DiOS, 2-acetamido-2-deoxy-3-O-(β -D-glucopyranosyluronic acid)-D-galactose; Δ Di4S, 2-acetamido-2-deoxy-3-O-(β -D-glucopyranosyluronic acid)-4-O-sulpho-D-galactose; Δ Di6S, 2-acetamido-2-deoxy-3-O-(β -D-glucopyranosyluronic acid)-6-O-sulpho-D-galactose; N.S., not significant.

individual strands of HA that are stabilized by the HC modification, which may be important in inflammatory cell recruitment (note: the term “HA cable” was first introduced by Carol de la Motte and her coinvestigators to distinguish HA structured in long-stranded configurations as opposed to the more diffuse HA staining patterns on the cell surface and in the pericellular matrix of smooth muscle cell cultures and inflammatory bowel disease tissue sections) (11). Regardless of the mechanism whereby the HC modification

promotes leukocyte adhesion to HA matrices, our data, and those of others, indicate that HC modification of HA regulates inflammatory events by promoting leukocyte adhesion, and possibly their long-term retention and activation in CF airways.

Our studies extend the limited available observations that have already been published on examining HA pathobiology in the progression of CF airway disease or as a marker of clinical exacerbation (17). In fact, HA serum levels have not been found to be impacted by advanced lung

disease in pediatric and adult patients with CF (28). Locally in the lung, HA at one time was reported to be the only detectable GAG in CF (29), although other sugars have been subsequently identified, which may also play an important role in CF pathophysiology (30). Interestingly, there have been studies to tie directly dysfunctional CFTR activity and HA activity in CF. In these studies, the investigators demonstrate that CFTR is a channel through which HA synthases extrude HA into the extracellular space (31).

The interrelationship between CFTR and HA synthases was based on the following observations: (1) small interfering RNA (siRNA) knockdown of CFTR in multidrug resistance protein 5 (MRP5) deficient mouse fibroblasts resulted in the inhibition of HA export; (2) inhibition of these cells with N-(2-naphthalenyl)-((3,5-dibromo-2,4-dihydroxyphenyl)methylene) glycine hydrazide (GlyH-101, an inhibitor of chloride export by CFTR) resulted in decreased HA export; (3) activation of CFTR with CFTR activator 06 (CFTR_{act}-06) stimulated HA export; and (4) the export of fluorescent HA oligosaccharides loaded into ΔF508 human epithelial cells demonstrated defective export compared with control cells (31). Using a competitive inhibition ELISA-like assay on guanidine extracted HA, this group further demonstrated elevated levels of HA in the bronchial secretions (sputum) of patients with CF compared with patients with bronchitis. The disparity between these published results and ours might be the result of several technical and biological differences between the studies, including: (1) the different methodology employed; (2) the means of normalization; (3) the mode of sputum collection and storage; (4) the age of the subjects; (5) the stage of airway disease; and/or (6) the CFTR mutation involved. Nevertheless, a separate, earlier study reported that CF sputum contained low levels of GAGs (32), thus consistent with our findings. Altogether, these seemingly disparate observations regarding HA in CF sputum attest to the need for further investigations to clarify this important issue.

HA was clearly detectable in non-CF disease control sputum (Figures 6A–6C),

and has been reported to be induced in the bronchial secretions after occupational and environmental exposures to noxious substances, and several pulmonary diseases, including chronic obstructive pulmonary disease, asthma, sarcoidosis, idiopathic pulmonary fibrosis, and bronchiolitis obliterans, and in the airway secretions of smokers and individuals having undergone a lung transplant (3). One study indicated that the origin of HA in airway secretions was from the serous, but not mucous cells of the human nasal and tracheobronchial submucosal glands (33). Our observation that the HA degrading capacity of the CF sputum analyzed in this study was negligible (Figures 6D and 6E) indicates that the depolymerization of HA matrices in these samples probably had already occurred well before collection. The mechanisms whereby HA can be degraded may include capture by phagocytic inflammatory cells in the airway lumen, cleavage by reactive oxygen species produced by neutrophils (34), or by hyaluronidases produced by pathogens that colonize CF airways (34).

Because HA levels were significantly lower in CF sputum, it suggests that the absence of this GAG might have adverse effects on mucus hydration, rheology, and the clearance of pathogens. Recent reports that HA supplementation with nebulized hypertonic saline improved clinical outcomes in patients with CF (13–17) and decreased inflammation in a CF mouse model (18). These studies suggest that one of the mechanisms for the therapeutic effect of nebulized HA may simply be that it is replacing an important structural component of normal mucus that is deficient in CF. Nebulized HA is purified

high-MW HA and does not have significant HC substitution. Thus, this type of high-MW HA likely acts more as a hydration molecule and, because of its rheological properties, likely acts as a lubricant to help expel sputum. As our current study demonstrates, the HA produced by CF lung tissue is highly substituted with HC, which then attracts and activates inflammatory cells. Our interpretation is that the quality of the high-MW HA, not necessarily its quantity, is likely to explain the beneficial effects of purified high-MW HA nebulized into the pulmonary tracts, as opposed to intrinsically produced in CF tissues leading to or exacerbating a proinflammatory condition.

In summary, these data support the hypothesis that HC-HA modification in CF lung tissue may have the potential to enhance inflammatory processes by engaging infiltrating inflammatory cells, the aggregation of which in lung tissue will likely result in the degradation of this hydrating GAG and exacerbate a chronic inflammatory cycle. ■

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