Identification of Glycosaminoglycans in Human Airway Secretions

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Glycosaminoglycans (GAGs), known to be present in airway mucus, are macromolecules with a variety of structural and biological functions. In the present work, we used fluorophore-assisted carbohydrate electrophoresis (FACE) to identify and relatively quantify GAGs in human tracheal aspirates (HTA) obtained from healthy volunteers. Primary cultures of normal human bronchial epithelial (NHBE) and submucosal gland (SMG) cells were used to assess their differential contribution to GAGs in mucus. Distribution was further assessed by immunofluorescence in human trachea tissue sections and in cell cultures. HTA samples contained keratan sulfate (KS), chondroitin/dermatan sulfate (CS/DS), and hyaluronan (HA), whereas heparan sulfate (HS) was not detected. SMG cultures secreted CS/DS and HA, CS/DS being the most abundant GAGs in these cultures. NHBE cells synthesized KS, HA, and CS/DS. Confocal microscopy showed that KS was exclusively found at the apical border of NHBE cells and on the apical surface of ciliated epithelial cells in tracheal tissues. CS/DS and HA were present in both NHBE and SMG cells. HS was only found in the extracellular matrix in trachea tissue sections. In summary, HTA samples contain KS, CS/DS, and HA, mirroring a mixture of secretions originated in surface epithelial cells and SMGs. We conclude that surface epithelium is responsible for most HA and all KS present in secretions, whereas glands secrete most of CS/DS. These data suggest that, in diseases where the contribution to secretions of glands versus epithelial cells is altered, the relative concentration of individual GAGs, and therefore their biological activities, will also be affected.

Keywords: airway; glycosaminoglycan; mucus

Mucus secretion and mucociliary clearance are integral components of airway host defenses (1, 2). Any change, either in quality or quantity of mucus, may result in the development of mucociliary dysfunction associated with diseases such as asthma, chronic bronchitis, and/or cystic fibrosis (3–6). The composition of mucus is complex, and includes water, ions, proteins, glycoproteins, lipids, and glycosaminoglycans (GAGs) (7–9), which, with the exception of hyaluronan (HA), are covalently bound to core proteins to form complex macromolecules, such as proteoglycans (PGs) (i.e., fibromodulin, lumican, and decorin). Although the glycoproteins, particularly mucins, have been extensively studied (10, 11), little is known about the GAG components of mucus.

GAGs are linear polysaccharides composed of a variable number of repeating disaccharide units. Based on the repeating disaccharide, GAGs are classified into four groups, including HA, chondroitin sulfate (CS)/dermatan sulfate (DS), keratan sulfate (KS), and heparan sulfate (HS). With the exception of

HA, which is synthesized by HA synthases at the plasma membrane, the synthesis of GAGs takes place in the golgi apparatus, and requires a host of nucleotide sugar transporters and glycosyltransferases. In extracellular matrix (ECM), PGs and GAGs, in addition to their structural role, participate in many physiologic processes, such as cell migration and differentiation (12, 13), antigen recognition (14), cell adhesion and communication (15, 16), and wound healing (17). In the lungs, GAGs are distributed in the interstice (18), in the subepithelial tissue and bronchial walls (19–21), and in airway secretions (9, 22). GAGs present in airway mucus have additional biological functions. For example, HA regulates tissue kallikrein catalytic activity (23) by immobilizing this enzyme in the airway surface, (22), whereas soluble HA increases ciliary beat frequency (24). Another GAG, CS, has been suggested to contribute to the insolubility of mucus in cystic fibrosis (6), whereas KS polymers have been shown to inhibit matrix metalloproteinase-2 and activate matrix metalloproteinase-9 (25). Despite the variety of processes in which GAGs can participate, the composition and concentration of GAGs and the GAG-associated PGs in airway secretions is not clear; therefore, the roles they may play in health and disease are mostly unknown.

In this work, we used fluorophore-assisted carbohydrate electrophoresis (FACE), a technique that allows identification and quantization of these macromolecules (26) to determine the GAGs present on human bronchial secretions. Human tracheal aspirates (HTA) obtained from healthy volunteers were used for assessment of GAG content *in vivo*, and the potential differential contribution between airway epithelial surface and submucosal gland (SMG) cells to the GAGs found in HTA samples was investigated *in vitro* by using primary cultures of normal human bronchial epithelial (NHBE) and SMG cells. In addition, confocal microscopy served to determine the localization of individual GAGs in human tracheal tissue sections and in NHBE and SMG cell cultures.

MATERIALS AND METHODS

All materials were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified.

HTA

HTA were obtained following a protocol approved by the University of Miami Institutional Review Board. The samples were collected from patients undergoing general anesthesia for elective surgery indicated for nonpulmonary reasons, as previously described (27). Briefly, secretions were collected by instilling 4 ml saline solution through a suction catheter that was advanced through an endotracheal tube into the trachea, followed by immediate suctioning. The samples were centrifuged at $500 \times g$ for 5 min to remove cells, followed by $16,000 \times g$ for 20 min at 4°C. The second supernatant was stored at –20°C until use (27). Three aliquots containing the same amount of proteins (0.5 mg each) were digested with proteinase K (125 µg/ml for 2 h at 60°C), and centrifuged at $5,000 \times g$ for 5 min. Supernatants were filtered using a Nanosep 3K (Pall Corporation, Ann Arbor, MI) to remove salts and other small molecules from culture media. The samples were freeze-dried and prepared for FACE analysis. Triplicate samples from four different individuals were used for these experiments.

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FACE

FACE was performed as previously described (28–30). Briefly, samples were subjected to digestion with glycosidases as follows: for HA and CS/DS, pellets were resuspended in 100 μ l of 0.1 M ammonium acetate, pH 7, and digested with 10 mU of chondroitinase ABC (ABC; ICN Biomedicals, Irvine, CA) and 10 mU of hyaluronidase from *Streptococcus dysgalactiae* (Seikagaku Corp., Tokyo, Japan) for 3 h at 37°C. For HS, the pellets were resuspended and digested with 20 mU of heparitinase 1 from *Flavobacterium heparinum* (Hep1; Seikagaku) in digestion buffer (0.1 M ammonium acetate, 10 mM calcium acetate, pH 7) for 1 h at 37°C. For KS, another set of dried pellets was digested overnight at 37°C with 5 mU of keratanase II (KII), from *Bacillus* sp. (KS36), and 5 mU of endo- β -galactosidase (EB) from *Escherichia freundii*, (both from Seikagaku) in 0.1 M ammonium acetate, pH 6. To assess fucose (fuc) substitution in KS, half of each sample (50 μ l) was further digested overnight at 37°C with 10 μ U of α -fucosidase (Prozyme, San Leandro, CA) in 50 mM sodium acetate (pH 5), and then dried in a vacuum concentrator. The dried samples and standards were fluorotagged with 2-aminoacridone (Molecular Probes, Carlsbad CA), as previously described (28, 30). A total of 5 μ l of derivatized digestion products was electrophoresed on MONO composition gels with MONO running buffer (Prozyme). The samples were electrophoresed at 4°C for 80 min at 500 V, and bands were identified by co-electrophoresis of HA and CS/DS disaccharide standards (Seikagaku) and 6-N-acethyl-galactosamine and 4-N-acethyl-galactosamine (EMD Biosciences Inc, La Jolla, CA). HS product standards were a mix of nonsulfated, monosulfated, disulfated, and trisulfated HS disaccharide standards (Seikagaku). For KS analysis, digestion products from bovine KS (generously provided by Dr. Anna Plaas, University of South Florida) and monosaccharide standards were used as described previously (30).

Digestion Product Quantification

A set of 2-aminoacridone-derivatized standards containing HA disaccharides (Δ DiHA) (250 pmol), nonsulfated disaccharides (Δ Di0S; 125 pmol), chondroitin 6-sulfate disaccharide (Δ Di6S; 60 pmol), chondroitin 4-sulfate disaccharide (Δ Di4S; 25 pmol), and chondroitin trisulfate disaccharide (Δ Di2,4,6S; 10 pmol) (STD 1) or Δ DiHA (15 pmol), Δ Di0S (30 pmol), Δ Di6S (60 pmol), Δ Di4S (125 pmol), or Δ Di2,4,6S (250 pmol) (STD 2) was included in each gel to calibrate the fluorescent intensity curve. The fluorescent images were captured with a ChemiDoc XSR camera (Bio-Rad, Hercules, CA) on a 304 nm transilluminator. The bands were analyzed and the intensities were corrected using a local background subtraction method using Quantity One software (Bio-Rad). A regression curve was used to calculate the concentration of derivatized product per band. The fluorescence of each band was plotted versus disaccharides (pmol) in standards (STD 1 and STD 2), and the total concentrations of individual GAGs were determined by adding the concentration of each band (digestion product) and expressed in nmol/mg protein as mean $\pm$ SEM.

Cell Cultures

Isolation procedure. Human tracheas and main bronchi from donor lungs rejected for transplantation were obtained through the University of Miami Life Alliance Organ Recovery Agency, with approval from the local institutional review board. The trachea and main bronchi were opened at the membranous portion, and the mucosa was dissected off the cartilage. Mucosal strips were digested with 0.05% protease (type 14) in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Carlsbad, CA) and incubated over night at 4°C for 24 h. NHBE cells were released by vigorous shaking, and cells were harvested by centrifugation. As previously described (27, 31, 32), the remaining tissue was further digested for another 24 h in 0.01% dispase in DMEM supplemented with antibiotic and scraped to release SMG cells.

NHBE cells grown at the air-liquid interface. Human tracheobronchial epithelial cells obtained after protease digestion were counted and their viability was determined by trypan blue exclusion (viability was always > 95%). A total of 1×10^6 cells were plated on collagen (rat tail collagen, 0.05% in 0.2% acetic acid)-coated plastic dishes (100 mm; Corning Costar Corporation, Cambridge, MA), grown to confluence in bronchial epithelial growth medium, yielding undifferentiated airway epithelial cells, and passaged after enzyme dissociation with trypsin (7). From passage 1, 5×10^5 cells were plated onto 24-mm

transwell clear culture inserts (Corning) coated with human placental collagen. The culture medium (air-liquid interface [ALI] medium) contained 50% DMEM and 50% Lechner and LaVeck basal medium supplemented with insulin (5 μ g/ml), hydrocortisone (0.072 ng/ml), epidermal growth factor (0.5 ng/ml), triiodothyronine (6.5 ng/ml), transferrin (10 μ g/ml), epinephrine (0.6 μ g/ml), phosphoethanolamine (0.5 μ M), ethanolamine (0.5 μ M), bovine pituitary extract (1% vol/vol), BSA (0.5 mg/ml), CaCl_2 (0.08 mM), trace elements, penicillin/streptomycin (100 μ g/ml), and retinoic acid (1 μ M) (31). Cells were grown in an incubator at 37°C in humidified air supplemented with 5% CO_2 . The apical surface of the cells was exposed to air as soon as they reached confluence. Conditions were maintained until the cells reached full redifferentiation (as assessed by the presence of beating cilia in the cultures, $\sim$ 4 wk) and used for the experiments described subsequently.

SMG cells. Cells were processed as previously described (32).

Sample Collection

Supernatants and basolateral (BL) media from SMG cells and PBS apical washes, as well as BL media from NHBE cultures, were collected. The cell layers were lysed with 4 M guanidinium chloride, 1% Triton X 100 (lysis buffer). All samples collected were stored at -20°C until FACE analysis. Triplicate samples from four different lung donors were used. Three aliquots from each compartment (apical, BL, and cells lysates) obtained from the equivalent of 1 mg protein in the cell lysates (for HA, CS/DS, and HS) and 0.5 mg of protein in cell lysates (for KS) were processed for FACE as described above.

Immunofluorescence

Human tracheal tissue sections and cell culture inserts obtained from two different lung donors were fixed in paraformaldehyde 4% in PBS, pH 7.4. Before processing, tissues were deparaffinized, and each filter was cut into four pieces for the various stains. Nonspecific binding was blocked with 0.2% BSA in Hanks' balanced salt solution for 2 h at room temperature, followed by incubation for 16 h at 4°C with primary antibodies to CS, KS, or HS (all from Seikagaku), or biotinylated hyaluronic acid binding protein (bHABP; EMD Biosciences) diluted in blocking solution as follows: mouse IgM anti-chondroitin sulfate (5 μ g/ml), mouse IgG anti-KS 5D4 (5 μ g/ml), FITC-labeled mouse IgG anti-HS EH4 (5 μ g/ml), and bHABP (2 μ g/ml). Visualization was achieved using Alexa 555 labeled anti-mouse IgG (for KS, 4 μ g/ml; Molecular Probes), rhodamine-labeled anti-mouse IgM (for CS, 2 μ g/ml; KPL, Gaithersburg, MD), and FITC-labeled avidin (for HA, 2 μ g/ml; Vector, Burlingame, CA). Controls (tissue sections and filters) were obtained by pretreatment with KII (10 mU), hyaluronidase from *Streptomyces hyaluronictus* (100 TRU), ABC (20 mU), and/or Hep1 (30 mU/ml), all from Seikagaku.

Statistical Analysis

Data were expressed as mean $\pm$ SEM. Statistical inference of the data was estimated by one-way analysis of variance followed by the Tukey-Kramer honestly significant difference test. Significance was accepted at $p < 0.05$.

RESULTS

FACE Analysis of Normal HTA

To identify the GAGs contained in airway secretions, HTA samples were processed as described in MATERIAL AND METHODS. After *Streptococcus dysgalactiae* hyaluronidase and ABC digestion, Δ DiHA and both nonsulfated (Δ Di0S) and sulfated CS/DS disaccharides (Δ Di6S and Δ Di4S) were found in these samples (Figure 1A). In contrast, no digestion products were detected in the samples treated with Hep1 (Figure 1B), indicating that HS, if present, was at a concentration below our detection limit of 20 pmol/mg protein. To assess the presence of KS, HTA samples were digested with KII and EB as described in MATERIALS AND METHODS. We detected KS monosulfated products (KS-MSP: galactose [gal]-glcNAc [6S]; glcNAc [6S]-gal) and disulfated products (KS-DSP: gal [6S]-glcNAc [6S]), which are shown in Figure 1C. We did not analyze the few faint bands running

Figure 1. FACE analysis of glycosaminoglycans (GAG) from human tracheal aspirates (HTA). Samples were digested with: (A) chondroitinase ABC (ABC) and *Streptococcus dysgalactiae* hyaluronidase, (B) heparitinase1 (Hep1) or (C) keratanase II (KII) and endo- β -galactosidase (EB). Products were fluorotagged and electrophoresed as described in MATERIALS AND METHODS. HA and CS/DS bands were identified by co-electrophoresis with the standards (STD): Glucose (glu); hyaluronan (HA) disaccharides (Δ DiHA); nonsulfated chondroitin disaccharides (Δ DiOS); N-acetyl galactosamine 6-sulfate (6SgalNAc); N-acetyl galactosamine 4-sulfate (4SgalNAc); monosulfated chondroitin disaccharide (chondroitin 6-sulfate disaccharide [Δ Di6S], chondroitin 4-sulfate disaccharide [Δ Di4S], and chondroitin 2-sulfate [Δ Di2S]), and trisulfated chondroitin disaccharide (Δ Di2,4,6S). HTA lanes 1 and 2 represent two different donors. Heparan sulfate (HS) products were identified with a mix of nonsulfated, monosulfated, disulfated, and trisulfated HS disaccharide standards (Δ Di-HS). Keratan sulfate (KS) products are indicated as monosulfated (KS-MSP; Gal [fuc]N-acetyl glucosamine [GlcNAc] [6S], Gal-GlcNAc [6S] and GlcNAc [6S]-Gal, from top to bottom) and disulfated (KS-DSP) products. Monosaccharide standards (MS): mannose (man), fucose (fuc), galactose (gal), glcNAc, gal 6-sulfate (gal6S) were used as mobility markers, whereas products from bovine KS digestion (STD-KS) were used as structural standards. *Bands are unidentified KS products obtained after digestion. O/E, overexposed image. (D) Values are the mean $\pm$ SEM of triplicate samples from three different individuals.

above the monosaccharide standards (presumably nonsulfated polylactosamine products [30]). We also detected another band migrating between gal and fuc. A similar band reported previously (33) has been shown to be the fucosylated monosulfated product, gal (fuc)-glcNAc (6S, see below). As shown in Figure 1D, KS disaccharides were the most abundant component of normal human tracheal secretion (55 ± 50 nmol/mg protein), followed by CS/DS (2.0 ± 0.2 nmol/mg protein), and HA (0.60 ± 0.03 nmol/mg protein). These KS products are likely derived not only from PG-associated KS, but also from KS chains present in other glycoproteins, such as mucins.

Immunolocalization of GAGs in Human Airways

Specific antibodies against CS, HS, and KS and bHABP for HA were used for localization of GAGs in human trachea sections and images were analyzed by confocal microscopy. Figure 2, A2, shows that both HS and CS are distributed in the ECM, located between glands; however, only CS staining was found inside some SMG cells. A slight signal for CS was observed apically in the airway epithelium, whereas HS was only expressed in subepithelial tissue, colocalizing with CS (Figure 2, C2). Whereas a strong staining for HA was found in glands, no signal was

detected for KS (Figure 2, A4). In contrast, both HA and KS were detected apically in the surface epithelium (Figure 2, C4), but without colocalizing. In addition, HA was found widely distributed in the subepithelium, unlike KS immunoreactivity, which was limited to the basal lamina (Figure 2, C4). These results are consistent with the FACE analysis findings, confirming that all GAGs detected by enzymatic digestion in HTA (KS, CS, and HA) are visualized in glands and surface epithelial cells, suggesting that these cells are responsible, at least in part, for the GAGs present in the airway secretions of healthy volunteers. In addition, KS immunoreactivity further confirms that fragments obtained with KII and EB were sulfated polylactosamine (KS) products.

SMG and NHBE Cells Express HA and CS/DS

Primary cultures of NHBE cells, grown at the ALI, and SMG cells were used to determine the contribution of the surface epithelium and SMG to total GAGs present in airway secretions. In addition, a potential polarized GAG secretion was assessed by quantifying GAGs in apical and BL secretions. As shown in Figures 3A and 3B, both cell types produced HA (Δ DiHA). A slight difference in HA secretion between apical (0.73 ± 0.30 nmol/mg cell lysate protein; $P < 0.05$) and BL compartments was observed in SMG cells (0.96 ± 0.30 nmol/mg cell lysate protein; $P < 0.05$; Figures 3C and 3D). In NHBE cells, HA was also secreted into the apical domain (1.23 ± 0.30 nmol/mg cell lysate protein; $P < 0.05$), but higher amounts of HA were released into BL compartment (3.42 ± 0.30 nmol/mg cell lysate protein; $P < 0.05$; Figures 3C and 3D). The concentrations observed in the cell lysates are the sum of intracellular and cell membrane-associated GAGs that were not released during PBS washes. SMG cultures produced both nonsulfated (Δ DiOS) and sulfated chondroitin/dermatan disaccharides (Δ Di6S and Δ Di4S), whereas only sulfated CS/DS disaccharides were detected in NHBE cells. CS/DS levels were much higher in SMG cultures (1.12 ± 0.20 nmol/mg cell lysate protein; $P < 0.05$) than in NHBE cells (0.03 ± 0.01 nmol/mg cell lysate protein; $P < 0.05$; Figures 3C and 3D). As expected, a band corresponding to glucose was visualized in our cell cultures (Figures 3A and 3B). Cell lysate fractions, in addition to intracellular products, likely contain GAGs that are associated with membrane proteins, and therefore were not released during washings and were not present in BL media. Unidentified bands were also present in the cell lysates from both cell types (Figures 3A and 3B), as has been previously reported (29). Figures 3C and 3D show the levels of HA and CS/DS (expressed as the sum of all bands) secreted into the apical and BL compartments. The differential expression suggests that glands are the main contributors to CS/DS, whereas surface epithelial cells are responsible for most HA present in airway mucus in normal conditions. This observation was confirmed by confocal microscopy (see below) and agrees with the staining intensity seen in tracheal sections.

NHBE Cells Produce and Secrete KS at the Apical Surface

As described in the Methods section, a separate digestion with KII and EB was used to determine the expression of KS in both SMG and NHBE cell cultures. As shown in Figure 4A, KS was not detected in SMG cultures. Figure 4B depicts large quantities of highly sulfated polylactosamine products consistent with KS, found in NHBE cultures that were secreted into the apical surface (13.80 ± 0.90 nmol/mg protein $P < 0.05$). The major bands identified were the monosulfated (MSP) products of gal-glcNAc (6S) and glcNAc (6S)-gal. No KS products were detected in the BL medium, indicating a highly polarized secretion pattern, in agreement with the observed distribution in tracheal tissue sections, that showed KS immunolabeling at the apical surface

A *Figure 2.* Immunolocalization of GAGs in human airways. Human trachea sections were double labeled with mouse anti-CS and FITC-labeled mouse anti-HS (column 2), or with mouse anti-KS and biotinylated HA binding protein for HA (column 4). Specific staining was visualized with Alexa555 conjugate secondary antibody (for KS), FITC-labeled avidin (for HA), and rhodamine conjugate secondary antibody (for CS). Confocal microscopy images show specific fluorescence in submucosal glands (SMG) (A and B) and airway epithelium (C and D). Controls were digested with ABC and heparitinase 1 (Hep1) (B2 and D2) or with KII and hyaluronidase from *Streptomyces hyaluronictus* (HYAL) (B4 and D4), as described in MATERIAL AND METHODS. Differential interference contrast images are shown in columns 1 and 3. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI).

of epithelial cells. Similar to HTA samples, a band previously reported as a fucosylated product of KS (30) was observed in the apical washes of NHBE cells. The identity of this band was confirmed by further digesting half of the samples with α -fucosidase. This treatment resulted in the disappearance of the band (arrow in Figure 4C) while the signals corresponding to fuc and to the MSP gal-glcNAc (6S) increased in intensity (Figure 4C). The presence of fucosylated products gal (fuc)-glcNAc (6S) confirms that the characteristics of KS secreted by these cultures are in agreement with our findings in HTA samples, thus suggesting that, in physiological conditions, cells from the surface epithelium are likely the major source of KS in airway secretions.

Immunolocalization of GAGs in SMG and NHBE Cultures

To assess the specific localization of individual GAG found by FACE, bHABP for HA and specific antibodies against CS, HS, and KS were used for detection by confocal microscopy as described in Methods. As shown in Figure 5, HA and CS were co-localized in SMG cultures and seem to be synthesized by the same cells. No KS or HS staining was observed, consistent with FACE analysis and trachea immunolabeling. Consistent with tissue section's findings CS was present at the surface of NHBE cells while HS was not visible (Figure 6A). A strong staining for HA was found distributed in the apical pole and in the ciliary

border of ciliated cells (Figure 6C). KS was detected at the apical surface on top of the cilia (Figure 6C) and separated from HA (see z-reconstruction in Figure 6C). Altogether, these findings corroborate the FACE results, pointing toward SMG as the main source of CS/DS, and surface epithelial cells as those responsible for most HA and all KS present in airway secretions.

DISCUSSION

Until recently, studies on GAGs and glycan-associated components of mucus in lungs were based on the use of biochemical and chromatographic methods, most of which relied on lectins to specifically recognize oligosaccharide products (34). Lately, with the availability of technologies such as cloning and expression of synthases and transferases, specific antibodies, and FACE, new approaches are being used to study the expression and secretions of mucins and PGs in airway mucus.

In the present study, FACE was used to identify and quantify GAGs in normal human airway secretions. Human trachea tissue sections, NHBE, and SMG cultures were used to differentiate the contribution of the surface epithelium from the SMG to the GAGs identified in human secretions.

We found that KS was the most abundant GAG present in the mucus layer, and its synthesis was limited to surface epithelial cells that released KS or KS-like molecules into the apical surface

Figure 3. FACE analysis of HA and CS/DS produced by primary cultures of human airway cells. Samples from apical (AP), cell lysate (CL), and basolateral (BL) compartments were collected and analyzed by FACE. (A) Normal bronchial human epithelial (NHBE) cells and (B) SMG. Disaccharide products content in AP (C) and BL (D) secretions. Values are expressed as nmol/mg cell lysate proteins. Each bar represents the mean $\pm$ SEM of triplicates samples from cultures from four different lungs. Standards are the same as in Figure 1. UB, unidentified bands.

in 4-fold greater amounts than any other GAG examined. KS products or highly sulfated polylactosamines are not found in free form, but, rather, associated to core proteins to form PGs, such as lumican, or other macromolecules, such as Muc1 (35).

Figure 4. KS from primary cultures of airway cell analyzed by FACE. SMG (A) and NHBE (B) cell cultures were digested with KII and EB, as described in MATERIALS AND METHODS. Digestion products are indicated as KS-MSP (Gal[fuc]GlcNAc[6S], Gal-GlcNAc[6S], and GlcNAc[6S]-Gal, from top to bottom) and KS-DSP. Bands were identified using monosaccharides standards (MS) as mobility markers: man, fuc, gal, glcNAc, gal6S, and with products derived from bovine KS digestion (KS-STD). (C) AP washes from NHBE cells with (+) or without (-) further digestion with α -fucosidase. White arrow indicates the disappearance of the fucosylated band.

Figure 5. Immunolocalization of GAG in SMG cells. The filters were double stained for HA (green) and CS (red) (HA/CS), or for HS (green) and KS (red) (HS/KS), using specific antibodies and biotinylated HA-binding protein as described in MATERIALS AND METHODS. Control filters were digested with HYAL and ABC (HYAL/ABC) or Hep1 and KII (Hep1/KII). Nuclei were visualized with DAPI.

The finding of KS in airway mucus is not novel. A keratanase-sensitive macromolecule was described in cat tracheal secretions (36) and in cultures of human and bovine airway epithelial cells (37, 38). As observed in nasal and auricular cartilages (33), and

Figure 6. Immunolocalization of GAG in NHBE cells. ALI cultures, labeled as described in MATERIALS AND METHODS, were used to visualize HS (green) and CS (red) (A) or HA (green) and KS (red) (C). Control filters were digested with ABC and Hep1 (B) or with HYAL and KII (D). Each panel includes the corresponding Z-axis reconstruction image at the bottom.

in cornea (39), we found that KS from HTA contains an α -(1-3)-fucosylation modification. The role of fuc modifications in KS remains unknown, although it has been suggested that this structural change may increase resistance to glycolytic enzymes (39, 40), whereas α -(1-3)-fuc-linked levels have been related to age (40). Confocal microscopy studies in tracheal tissue sections, as well as cell cultures, showed the same distribution, suggesting that in physiologic conditions, cells from the surface epithelium are major sources of KS in airway secretions.

We did not detect HS in either HTA or in primary cultures, indicating that, if present, its levels were below our detection limit of 20 pmol/mg of protein. The distribution of HS in trachea tissue sections was consistent with these findings: immunoreactivity was limited to lung parenchyma and basement membrane, as previously reported (41-43).

We also found, as shown before by others (44, 45), that tracheal secretions contain HA. Interestingly, we found that NHBE cells released HA into the apical and BL compartments. Because this distribution is also observed in tissue sections (in the surface epithelium and in the basal lamina), it is tempting to hypothesize that HA from epithelial cells can contribute to airway homeostasis at two different levels: apically, by immobilizing enzymes important in host defense (e.g., tissue kallikrein and lactoperoxidase) and increasing ciliary beat frequency (22, 24), and basolaterally, by being involved in functions such as organization of the ECM, wound healing, tissue repair, and maintenance of epithelial integrity (15, 46-51). The intensity of HA labeling and the tissue distribution in trachea, as well as in cell cultures, were in agreement with the FACE analysis. Interestingly, fluorescence for KS and HA in trachea and in NHBE cultures, although evident in the same apical compartment, did not colocalize, suggesting a clear separation between HA and KS in the surface of epithelial cells. These observations are in agreement with previous reports showing that KS is associated with gel-forming mucins (37), whereas HA is bound to the apical pole of airway epithelium (22), and suggest that KS may be mostly included in the gel phase and HA in the sol phase of airway secretions.

CS/DS were also present in HTA samples, and, according to the observations in cell cultures and confocal microscopy, epithelium and SMG are responsible for their synthesis and release into the bronchial lumen. These findings were expected, since early works (8) have shown that cells from SMG produced the CS PG decorin. SMG cultures produced higher amounts of CS/DS than NHBE cells. This observation is supported by the intensities and tissue distribution of CS immunolabeling in trachea and cell cultures. These results suggest that in normal conditions, glands are the main source of CS/DS secreted to the bronchial lumen. Here we found that cultures of SMG and NHBE cells secreted CS/DS, although with a different pattern. SMG cells produced both sulfated and nonsulfated CS/DS, whereas NHBE only secreted sulfated disaccharides, suggesting perhaps a differential regulation of sulfotransferases between these two cell types.

In summary, KS, HA, and CS/DS were present in human airway secretions, mirroring the mixture of GAGs found in primary cultures of NHBE and SMG. In addition, the cell distribution of individual GAGs assessed by confocal microscopy was consistent in human trachea tissue sections and in cell cultures. The data suggest that primary cultures of airway cells (NHBE grown at the ALI and SMGs) are suitable to study the regulatory mechanism associated with the expression and secretion of individual GAGs and PGs present in the airways.

In conclusion we found that KS is the most abundant GAG in airway secretions, and is synthesized by airway surface epithelial cells, whereas SMG were the main sources of CS/DS. These data suggest that, in diseases in which the contribution to secretions

of glands versus epithelial cells is altered, the relative concentration of individual GAGs, and therefore their biological activities, will also be affected.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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