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Hyaluronan Rafts on Airway Epithelial Cells*

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Many cells, including murine airway epithelial cells, respond to a variety of inflammatory stimuli by synthesizing leukocyte-adhesive hyaluronan (HA) cables that remain attached to their cell surfaces. This study shows that air-liquid interface cultures of murine airway epithelial cells (AECs) also actively synthesize and release a majority of their HA onto their ciliated apical surfaces to form a heavy chain hyaluronan (HC-HA) matrix in the absence of inflammatory stimuli. These matrices do not resemble the rope-like HA cables but occur in distinct sheets or rafts that can capture and embed leukocytes from cell suspensions. The HC-HA modification involves the transfer of heavy chains from the inter-α-inhibitor (IαI) proteoglycan, which has two heavy chains (HC1 and HC2) on its chondroitin sulfate chain. The transesterification transfer of HCs from chondroitin sulfate to HA is mediated by tumor necrosis factor-induced gene 6 (TSG-6), which is up-regulated in inflammatory reactions. Because the AEC cultures do not have TSG-6 nor serum, the source of IαI, assays for HCs and TSG-6 were done. The results show that AECs synthesize TSG-6 and their own heavy chain donor (pre-IαI) with a single heavy chain 3 (HC3), which are also constitutively expressed by human renal proximal tubular epithelial cells. These leukocyte adhesive HC3-HA structures were also found in the bronchoalveolar lavage of naïve mice and were observed on their apical ciliated surfaces. Thus, these leukocyte-adhesive HA rafts are now identified as HC3-HA complexes that could be part of a host defense mechanism filling some important gaps in our current understanding of murine airway epithelial biology and secretions.

Hyaluronan (HA)[2] is a linear, non-sulfated glycosaminoglycan that is made of repeating disaccharides composed of D-glucuronic acid and D-*N*-acetylglucosamine linked by β(1,4) and β(1,3) glycosidic bonds. HA is normally formed as an unmodified structure but is uniquely modified during inflammation (1–3). This modification involves the transfer of heavy chains (HC) from inter-α-inhibitor (IαI) to form HC-HA complexes mediated by tumor necrosis factor-α (TNFα)-induced protein-6 (Tnfaip6), also known as TNFα-stimulated gene-6 (TSG-6) (4). IαI is a proteoglycan produced by liver hepatocytes and released into the circulation. It is composed of the trypsin inhibitor bikunin with heavy chain 1 (HC1) and heavy chain 2 (HC2) that are covalently bound by ester linkages to bikunin via its single chondroitin sulfate chain. During inflammation, IαI can ingress into extracellular tissue spaces as a consequence of increased vascular permeability where it serves as a heavy chain donor. TSG-6 is expressed in stimulated cells and catalyzes the transfer of HC1 and HC2 from IαI to HA, forming the HC-HA complexes (5–10).

These HC-HA complexes have been identified in synovial fluids from patients with rheumatoid arthritis and osteoarthritis (11–14) and in bronchial secretions of asthmatics (16). Furthermore, the expression of TSG-6 is up-regulated in inflamed synovial tissue of arthritis and in lungs of asthmatics (16, 17). The expression of TSG-6 is among the genes up-regulated >4 fold in allergen-challenged patients (16) emphasizing the important role that TSG-6 has during inflammation. HC-HA matrices promote leukocyte adhesion in pathological conditions, including rheumatoid arthritis (13), idiopathic arterial pulmonary hypertension (18), and asthma (19).

During many pathological processes a variety of cell types synthesize leukocyte-adhesive HA structures on their cell surface with coalesced HA strands referred to as HA cables (20). These modified HA cables have structural information recognized by mononuclear leukocytes (20, 21). Leukocytes can bind to HA cables at 4 °C and rapidly phagocytose the matrix at a physiological temperature of 37 °C. Recently, we found that transfer of HCs to form these HA matrices resulted in more pronounced cable formation that promotes greater leukocyte adhesion (22). In all cell types studied to date, the leukocyte-adhesive HA cables synthesized by these cells remain attached to the cell surface. However, although airway epithelial cells (AECs) also form HA cables on their cell surface in response to a variety of inflammatory stimuli (23), we show in this study that they also actively release a majority of their leukocyte-adhesive HA structures into the extracellular space from their apical surface. These matrices do not resemble the HA cables but occur in distinct sheets, or rafts, of HA that are leukocyte-adhesive. Thus, a HA raft with HC-HA structure present in respiratory secretions raises a number of important questions regarding the role it has in inflammatory processes.

Experimental Procedures

Mice—All mice were maintained in the Biological Resource Unit of the Cleveland Clinic Lerner Research Institute in a temperature-controlled facility with an automatic 12-h light-dark cycle and were given free access to food and water. All animal

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[2] The abbreviations used are: HA, hyaluronan; HC, heavy chain; Iαl, inter-α-inhibitor; TSG-6, tumor necrosis factor-stimulated gene-6; AEC, airway epithelial cell; PTEC, proximal tubular epithelial cell; BAL, bronchoalveolar lavage; MDCK, Madin-Darby canine kidney.

protocols were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic. TSG-$6^{-/-}$ mice (24) were a generous gift from Dr. Tibor T. Glant (Rush University Medical Center, Chicago, IL), and 4–6-week-old female TSG-$6^{-/-}$ mice were used. Wild type controls on a BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, ME) and used for preparing airway epithelial cell cultures and for collecting bronchoalveolar lavage (BAL).

MDCK Basement Membrane—Basement membranes were previously generated by applying Madin-Darby canine kidney (MDCK) cells onto a reconstituted collagen substrate laid on the surface of the porous membranes of 12-well tissue culture transwell inserts (353180; Falcon) (23). We used this model for preparing vertical sections for microscopy. However, contraction of the collagen substrate by the AECs can create an imperfection in which some AECs can grow into the collagen layer as shown in the *lower right* of Fig. 3*D* and Fig. 1*B*. We have solved this problem by adding MDCK cells straight onto the porous membrane of the transwell insert. After reaching confluence, the MDCK cells were removed by detergent lysis using PBS with 0.5% IGEPAL® CA-630 (I8896; Sigma) leaving behind a native basement membrane on the transwell surfaces. The AECs grew more uniformly in the absence of the collagen layer (not shown) making a better culture model for differentiated AECs. However, the close attachment of the AECs to the transwell via the basement membrane, unlike the collagen substrate model, prevents detachment of the AEC layer for making vertical sections for microscopy.

Primary Cell Culture—Murine AECs were derived from mouse tracheas and cultured on the basement membrane-covered transwells in an air/liquid interface model as previously described (23). Apical washes were collected by gently washing the apical surface of AEC cultures with 200 μl of phosphate-buffered saline (PBS) per insert of a 12-well plate. Washes were then frozen at −80 °C until the time of use.

Fluorophore-Assisted Carbohydrate Electrophoresis—HA contents in the apical washes and in equivalent proportions of basal media from identical inserts were measured by fluorophore-assisted carbohydrate electrophoresis as previously described (25). The DNA contents from the identical inserts used in this experiment were measured at the end of their differentiation (day 10) and used for normalization purposes. In addition, the hyaluronan to chondroitin sulfate content ratios in the apical washes and basal media collected from each time point were used for further verification of our normalization.

Hyaluronidase Extraction of HCs from the HC-HA Complex—HCs were determined in BAL samples and in apical washes of day 10 air/liquid interface cultures of primary differentiated AECs by treatment with or without *Streptomyces* hyaluronidase (0.5 turbidity reducing unit (TRU)/ml; 100740–1; Seikagaku, East Falmouth, MA) overnight at 37 °C. The supernatants were then analyzed by Western blotting.

Collection of BAL—Mice were euthanized by using isoflurane or pentobarbitol. BALs were obtained by cannulating the trachea with a 24 gauge-feeding needle and lavaging the lungs with 600 μl of PBS. The typical BAL fluid returned was 400–500 μl. A 100-μl aliquot of the collected BAL was transferred to a glass slide via cytospin and left to air dry completely before processing for immunohistochemistry.

Western Blot Analysis—Samples were electrophoresed on 4–5% Mini-PROTEAN TGX gels (Bio-Rad) and blotted using Bio-Rad PVDF membranes and the Trans-Blot Turbo System. In Fig. 4, *A* and *B*, cell lysates were separated by 10% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). All blots were blocked for 1 h with 5% milk and probed overnight at 4 °C with antibodies against HC1 (SC-33944; Santa Cruz Biotechnology), HC2 (SC-21978; Santa Cruz Biotechnology), HC3 (SC- 21979; Santa Cruz Biotechnology), bikunin (a kind gift from Anthony Day, University of Manchester, UK), and IαI (A0301; Dako Cytomation, Denmark). Biotin-SP-conjugated donkey anti-goat IgG was used as secondary antibody (705–065-147; Jackson ImmunoResearch) to generate the blot in Fig. 7*B* (*HC-3*). Blots were incubated with secondary antibody for 1 h at room temperature followed by treatment with streptavidin horseradish peroxidase (HRP) (P0397; Dako Cytomation) for 1 h at room temperature. TSG-6 was assessed using a biotinylated anti-mouse TSG-6 antibody (BAF2326; R&D Systems, Inc.). Recombinant mouse TSG-6 (2326-TS; R&D systems, Inc.) was used as a positive control for the TSG-6 antibody. All blots were developed using the ECL Prime Western Blotting Detection Reagent (RPN2232, Amersham Biosciences), and detected protein band intensities were measured using ImageJ software (imagej.nih.gov).

Immunohistochemistry—Aliquots of BAL or apical washes from AECs were transferred to a glass slide via cytospins and air-dried completely. Samples were then fixed with 100% methanol at −20 °C for 10 min and air-dried for 1 h. Paraffin-embedded sections of lung tissue were previously fixed overnight in 10% formalin. The sections and methanol fixed cytospins of BAL and apical washes of AECs were rehydrated in PBS for 30 min and then blocked with 1% BSA in PBS. HA was labeled with a HA-biotinylated-binding protein (5 μg/ml; 385911; EMD Chemicals, Gibbstown, NJ) in blocking solution and then with streptavidin conjugated to Alexa Fluor® 488 (1:500, product S11223; Invitrogen). These slides were simultaneously incubated with a mixture of antibodies to HC1, HC2, and HC3 using Alexa Fluor® 594 donkey anti-mouse secondary (1: 200; product A11058; Invitrogen) or anti-CD44 (1:250; product C7923, Sigma) or anti-KDEL (PA1-013; Affinity Bioreagents, Golden, CO). Vectashield mounting medium with DAPI (H-1200; Vector Laboratories, Burlingame, CA) was applied to the apical surface of the samples followed by coverslip application before visualizing using fluorescent microscopy.

Polymerase Chain Reaction (PCR) Analyses—RNA was isolated using the RNAeasy kit (74104; Qiagen), and cDNA was prepared using Superscript First-strand Synthesis System (11904–018; Invitrogen). Primers used are listed in Table 1. The PCR conditions were 1 cycle at 94 °C for 3 min, 40 cycles of 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min followed by 1 cycle of 72 °C for 1 min. These conditions were used for all primers. PCR products were run on 3% ethidium bromide gels (E1510; Sigma) and 1% agarose gels (SeaKem® HGT-agarose, product 50041; Lonza).

Statistical Analysis—Data are presented as the mean ± S.E.; n is indicated in the figure legends in representative experiments.

TABLE 1
The sequences of the PCR amplification primers

Name of primers	Primer sequence	Expected PCR product
β-Actin	5′-GGTCATCACTATTGGCAACG-3′ 5′-ACGGATGTCAACGTCACACT-3′	133 bp
Heavy chain 1	5′-TTCTCAGCCCTTAGAGATGGTG-3′ 5′-GAGTGGCAACTTTGAGTCTATGG-3′	100 bp
Heavy chain 2	5′-GGTGATAGAGAATGATGCTGGA-3′ 5′-CAACCTTGGTGCCATAATACAG-3′	101 bp
Heavy chain 3	5′-CCCAGAAAGATTACAGGAAGGA-3′ 5′-TCGGTATGGACACCATCAATTA-3′	100 bp
TSG-6	5′-CTCCATATGGCTTGAACAAGCAGC-3′ 5′-ACCACCTTCAAATTCACATACG-3′	112 bp

Results

HA Rafts on Apical Surfaces of Murine AECs in Vivo and in Vitro—HA is located on the apical surfaces (*white arrows*) of murine AECs as distinct clumps or HA rafts (*green*) that can be seen both *in vivo* on a normal WT epithelium (Fig. 1*A*) and on an air/liquid day 10 AEC culture *in vitro* (Fig. 1*B*). Furthermore, the air/liquid AEC culture model closely resembles its *in vivo* counterpart, as previously shown (23). Thus, it represents a compatible model to study the HA rafts that murine AECs make.

Murine AECs Release the Majority of HA from Their Apical Surface—The apical surfaces of air/liquid AEC cultures were gently washed during their course of differentiation over 10 days and analyzed for HA (Fig. 2*A*) in comparison to that in basal media. The purpose of that was to be able to determine the time point where cells reached a steady state in HA production, which is day 10 after lift. HA contents were then measured in apical washes, basal media, and cell layers (Fig. 2*B*). The results indicate that the vast majority of the HA synthesized by murine AECs is shown to be on their surface (Fig. 1*B*), is loosely attached, and only small amounts are present in the basal medium. We did not see a significant difference in the HA synthesized apically between wild type and TSG-6 null AECs *in vitro*. This implies that the synthesis of this loosely attached HA on the apical surface of murine AECs, which also appears to be present *in vivo* (Fig. 1*A*), may be a normal process of airway epithelial biology, although it may be modified during periods of stress and inflammatory stimuli.

Murine AEC Cultures Release Leukocyte Adhesive HA Rafts from Their Apical Surface—HA cables are formed at the cell surface of a variety of cells including AECs (21) during inflammatory processes. These structures are known to function as a ligand for inflammatory cells (20). However, the majority of the apical HA produced by AECs can be released into the extracellular space as HA rafts. To investigate whether these rafts are also leukocyte-adhesive, we incubated the apical washes of day 10 AEC cultures with a human cell line of monocytic U937 cells for 1 h at 4 °C. Subsequently, an aliquot was transferred to a slide via cytospin and stained for CD44, a HA receptor. Multiple clusters of U937 cells were embedded in the HA rafts as shown in (Fig. 3*A*–*C*). Furthermore, similar clusters of U937 cells were observed when they were added to the apical surface of an AEC culture (Fig. 3*D*). This indicates that the HA rafts released by the AECs are also leukocyte-adhesive, similar to the HA cables that are known to form in other pathologies. However, in this case, they are not being formed in response to a pathological stimulus. The CD44 antibody used in all of our experiments is specific to human CD44 and stains only for CD44 on the cell surface of the human U937 cells and not on the murine epithelial cells. This explains the negative staining of CD44 on the cell surface of murine AECs.

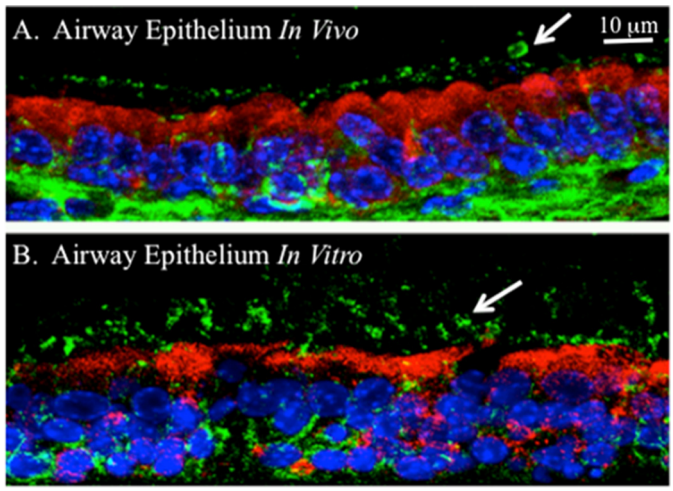

FIGURE 1. **HA rafts are loosely attached to the apical surface of murine airway epithelia *in vivo* and on AEC lift cultures *in vitro*.** One representative image out of three of the paraffin-embedded section of a murine airway epithelium *in vivo* (*A*) and a day 10 differentiated air/liquid AEC culture *in vitro* (*B*) were stained for HA with a fluorescent conjugate of a biotinylated HA-binding protein (*green*). Nuclei were stained with DAPI (*blue*) and for endoplasmic reticulum (*red*) with an antibody against KDEL. The *arrows* indicate the apical HA rafts. HA is also apparent in the basal areas with most of it in the underlying connective tissue *in vivo*. (magnification 63×, *magnification bar* of 10 μm).

HA Rafts Contain HC-HA Complexes—One possible explanation for the HA adhesiveness to leukocytes is due to a HA modification that normally occurs during inflammations, namely the transfer of HCs from IαI to form HC-HA complexes, which is mediated by TSG-6 (11, 14). Apical washes of day 10 AEC cultures were collected at the indicated times (2–48 h) after replacing the medium on day 10. They were then treated with *Streptomyces* hyaluronidase (specific for HA) and analyzed for HCs by Western blots (Fig. 4*A*). The accumulation of HCs at ~85 kDa over the time course is apparent. This band was not present in the absence of the hyaluronidase digestion as shown in the analysis of a day 12 apical wash (Fig. 4*C*). This provides evidence that monocyte binding is due to a large extent by the presence of HCs (11, 14) and that the HA rafts contain HC-HA complexes. Furthermore, TSG-6 was also present in the apical washes and absent in apical washes from TSG-6 null AECs (Fig. 4*B*). The absence of HC-HA in the TSG-6 null cultures is also apparent (Fig. 4*A*).

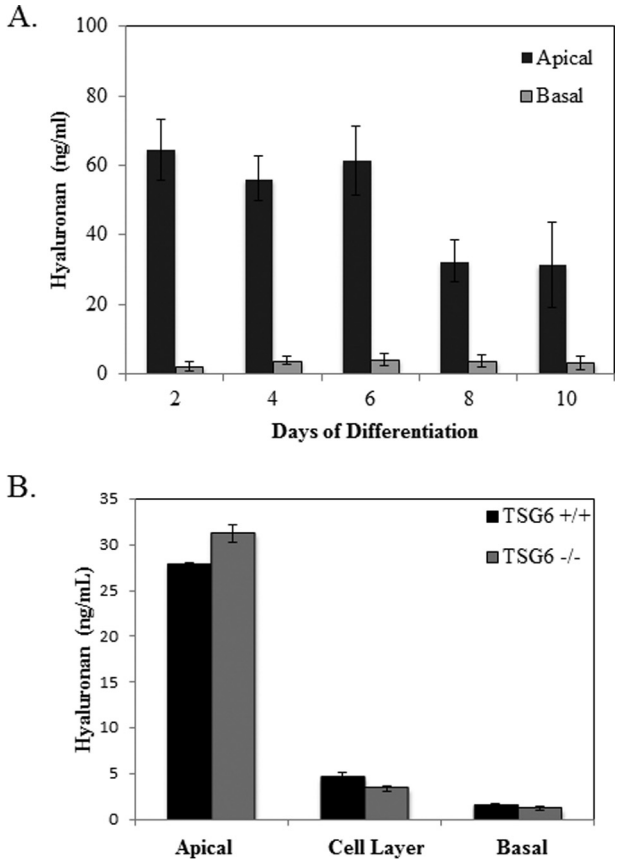

FIGURE 2. **Murine AECs release most of their HA from their apical surface.** *A*, the *bar graph* shows the amounts of HA determined by fluorophore-assisted carbohydrate electrophoresis in the apical washes (*black bars*) and equivalent proportions of volumes of basal media (*gray bars*) collected during the differentiation phase of WT AEC cultures. The values in the apical washes are statistically greater ($p < 0.01$) than those in the basal media from 2–10 days of differentiation with apical washes taken every 2 days. *B*, the *bar graph* compares the HA content in 3 compartments of WT and TSG-6 null AECs day 10 *in vitro*: apical wash, cell layer, and basal medium. This was done in replicates of six.

HA Rafts in Murine BAL Require TSG-6 for HC Transfer onto HA—To determine whether HA rafts are HC-HA complexes released by the airway epithelium *in vivo*, we analyzed BAL samples from a naïve WT mouse and found clusters of HA that are monocyte-adhesive similar to the HA rafts that AECs make *in vitro* as shown in (Fig. 5, *A–C*). Furthermore, these HA rafts lacked HCs in the absence of TSG-6 as shown in the BAL collected from a TSG-6 null mouse (Fig. 5, *D–F*), which is consistent with the fact that HC transfer onto HA requires TSG-6. Moreover, the size of the HA raft in the TSG-6 null BAL fluid was much smaller, suggesting that the lack of TSG-6 may have an effect on the synthesis or aggregation of HA. This is consistent with the significant decrease of pulmonary HA concentration measured in the BAL and lung tissues of TSG-6 null mice subjected to the ovalbumin asthma model (19). Furthermore, digesting the BAL collected from a naïve WT mouse with *Streptomyces* hyaluronidase resulted in the release of significantly more HCs into the gel that were detected on Western blots (Fig. 6, *A* and *B*), whereas HC bands were nearly absent (<10% of control) in BAL from a TSG-6 null mouse. This demonstrates the role of TSG-6 in mediating HC transfer onto HA in rafts located on the apical surface. Moreover, we have previously shown that TSG-6 can transfer all of the HCs from serum-derived HC-bikunin to HA1000K *in vitro* at early time points, whereas TSG-6 released some free HCs from HA1000K at later time (26). This may explain the observation of free HCs in the presence of TSG-6 when WT BAL was not treated with *Streptomyces* hyaluronidase.

Murine AECs Make Their Own HC3 Donor as HC3-bikunin—IαI is a proteoglycan that normally contains two HCs (HC1 and HC2). It is produced by the liver hepatocytes and released into the blood at relatively high concentrations. It serves as an HC donor for TSG-6 mediated transfer of HCs to HA during many inflammations. The AEC culture medium lacks serum. Therefore, the presence of HC-HA in the HA rafts from unstimulated AEC cultures suggests that AECs can make their own HC donor. From the three different HC subtypes (HC1–3) analyzed by RT-PCR, murine AECs express only HC3, as shown in Fig. 7*A*. Fig. 7*B* shows that the donor, which has a molecular mass ~132 kDa, contains HC3 and bikunin, which is equivalent to the pre-IαI complex. This donor is the same as the pre-IαI, made and secreted by the renal epithelium, which is composed of a bikunin with a HC3 covalently linked by an ester bond to the chondroitin-4-sulfate chain (27). Therefore, AECs synthesize their own HC3-bikunin in a manner similar to renal epithelial cells and are able to generate HC3-HA rafts without the requirement of a serum exudate produced during inflammations. We also analyzed the basement membrane deposited by MDCK cells, on which the AECs are cultured during their differentiation, by Western blotting for HC3 and TSG-6 protein levels as MDCK cells are also kidney epithelial cells. Protein levels of HC3 and TSG-6 were not detected (data not shown), confirming that the source of these two proteins is from the AECs and not the basement membrane.

TSG-6 Is Expressed by Unstimulated Murine AECs and Released Apically and Not Basally—The HC3-HA data in Figs. 5 and 6 provide evidence that AECs synthesize TSG-6 as well as their own HC3-bikunin. Therefore, we used total cellular RNA isolated from day 10-differentiated AECs and examined TSG-6 mRNA by RT-PCR. We used cellular RNA isolated from mouse dermal fibroblasts, known to express TSG-6 only when treated with TNFα (28), as our positive control (Fig. 8*A*). Furthermore, we analyzed Western blots of apical washes and basal media of AEC cultures for TSG-6 protein levels as probed with a biotinylated mouse TSG-6 antibody. This antibody detected the recombinant mouse TSG-6 at the expected molecular mass ~35 kDa (Fig. 8*B*). However, a larger band (~60 kDa) was detected in the apical washes of AECs (Fig. 8*B*). No band was detected in the basal medium, which indicates that neither the defined culture medium nor basally secreted products from the AECs contain reactive bands. The ~60-kDa band was present in the apical washes from WT AECs but absent in the apical washes of TSG-6 null AECs (Fig. 4*B*), indicating that the antibody likely detects TSG-6 in either a dimerized or modified form. Even though it requires further investigation, we think dimerization is more likely based on the ability of TSG-6 molecules to dimerize as shown in some *in vitro* studies (29, 30). Therefore, AECs can express TSG-6 on both transcriptional (Fig. 8*A*) and translational levels (Figs. 8*B* and 4*B*).

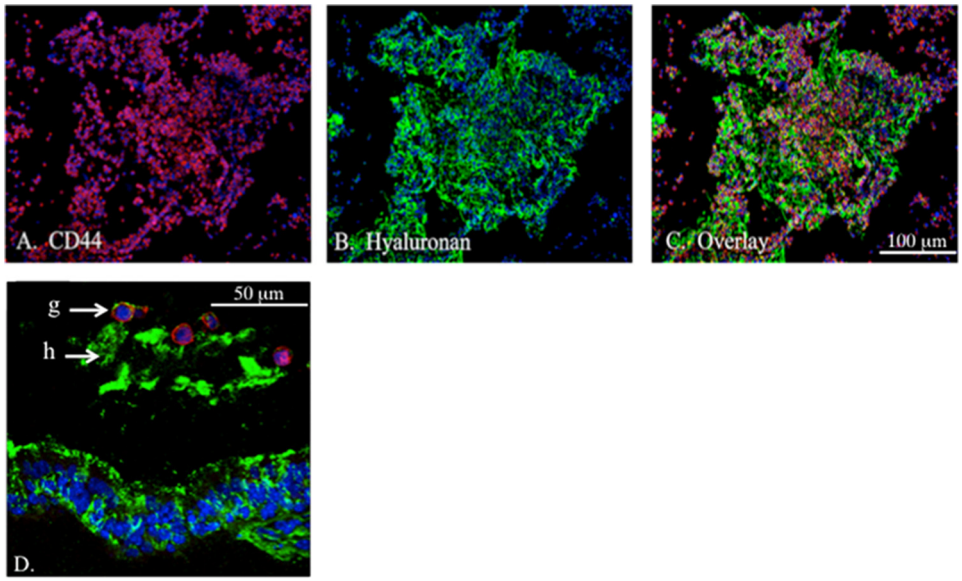

FIGURE 3. **Murine AECs release leukocyte-adhesive HA rafts from their apical surface.** *A–C*, monocytic U937 cells were suspended in the apical wash from a day 10 AEC culture for 1 h at 4 °C and subsequently cyto-spun onto a slide. HA was stained with a fluorescent conjugate of biotinylated HA-binding protein (*green*). U937 cells were probed with antibodies against human CD44 (*red*). This fluorescent micrograph shows multiple clusters of U937 cells embedded in HA rafts (magnification 20×, *magnification bar* of 100 μm). *D*, the micrograph of a section of a day 10 culture where AECs grew into the underlying type 1 collagen layer (*lower right corner*) on which MDCK cell membrane was laid (magnification 63×, *magnification bar* of 50 μm) shows an example of U937 monocytes (*g*) binding to the loosely attached HA raft (*h*).

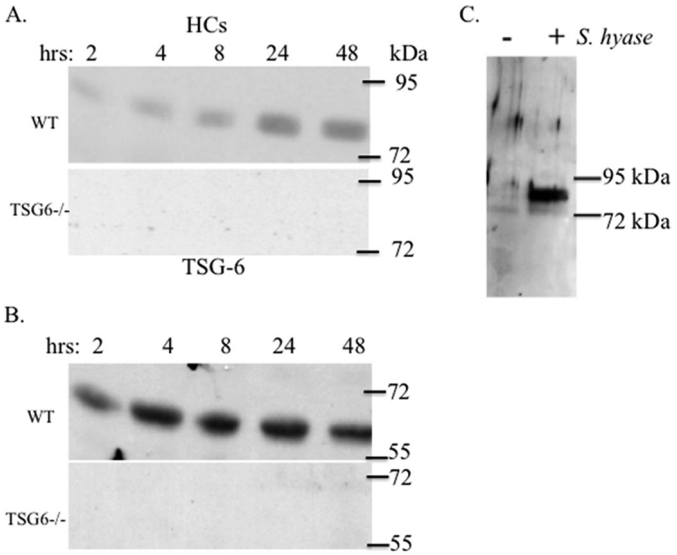

FIGURE 4. **Murine AECs produce their own HCs.** One representative out of two Western blots of apical washes of unstimulated AECs were collected at 2, 4, 8, 24, and 48 h after day 10 of their differentiation and treated with *Streptomyces* hyaluronidase then probed with a mixture of HC1, -2, and -3 antibodies (*A*) and re-probed with TSG-6 antibody (*B*). The absence of HC transfer and TSG-6 from a parallel experiment with AECs from TSG-6 null mice is apparent in *A* and *B* consecutively. *C*, one representative out of four Western blots of an apical wash collected on day 10 of differentiation, 48 h after the previous apical wash, treated without or with *Streptomyces* hyaluronidase (*S. hyase*). This blot was probed with an antibody against IαI.

Discussion

Cells under a variety of stresses synthesize HA cable-like structures on their cell surface. This was initially shown in cultures of smooth muscle cells from the colon stimulated with poly (I:C), a viral mimic, or by viral infection (20). Monocytes recognize and adhere to these modified HA structures (14, 21). These leukocyte-adhesive HA matrices are present in many pathological conditions, including lung biopsies from asthmatic patients (21), responses of smooth muscle cells to ER stress at physiological normal levels of glucose (31, 32), wound healing (33), idiopathic pulmonary hypertension (18), airway smooth muscle cells *in vitro* (32), airway interstitial cells in mouse asthma models (19), renal tubular endothelial stress (34, 35), and adipocytes in a diabetic rat model (36, 37). However, in this study we show that murine AECs behave differently than the other cell types reported so far. Besides making leukocyte adhesive HA cables that remain attached to the cell surface in response to a variety of inflammatory stimuli, they also actively release a majority of their HA matrices into the extracellular space from their apical surface (Fig. 2*A*). These matrices do not resemble the rope-like HA cables but occur in distinct sheets or rafts of HA that are leukocyte-adhesive (Fig. 3, *A–D*).

HA can be uniquely modified during inflammation by the transfer of HCs from IαI to form HC-HA complexes mediated by TSG-6. HC-HA matrices are leukocyte-adhesive as demonstrated in many pathological conditions. However, the lack of the TSG-6-mediated HC-HA matrix in normal cumulus cell-oocyte complexes causes female infertility in TSG-$6^{-/-}$ female mice (24). This demonstrates that HC-HA matrices can be part of a normal extracellular matrix as well as an inflammatory response to stimuli. AECs uniquely release leukocyte adhesive HC3-HA complexes from their apical surface into their extracellular space even in the absence of inflammatory stimuli (Fig. 4). HA rafts were not only observed in the air/liquid AEC culture model but also in the bronchoalveolar lavage of naïve mice (Fig. 5, *A–C*) that are HC3-HA complexes (Figs. 6 and 7) implying that these rafts are part of murine normal secretions and can have a role as a host defense mechanism. Leukocyte adhesion to HA rafts was also observed but to a lesser extent in the absence of a TSG-6 mediated HC transfer, as shown in Figs. 5, *D–F*, and 6, suggesting that HC attachment to the HA raft is not the sole

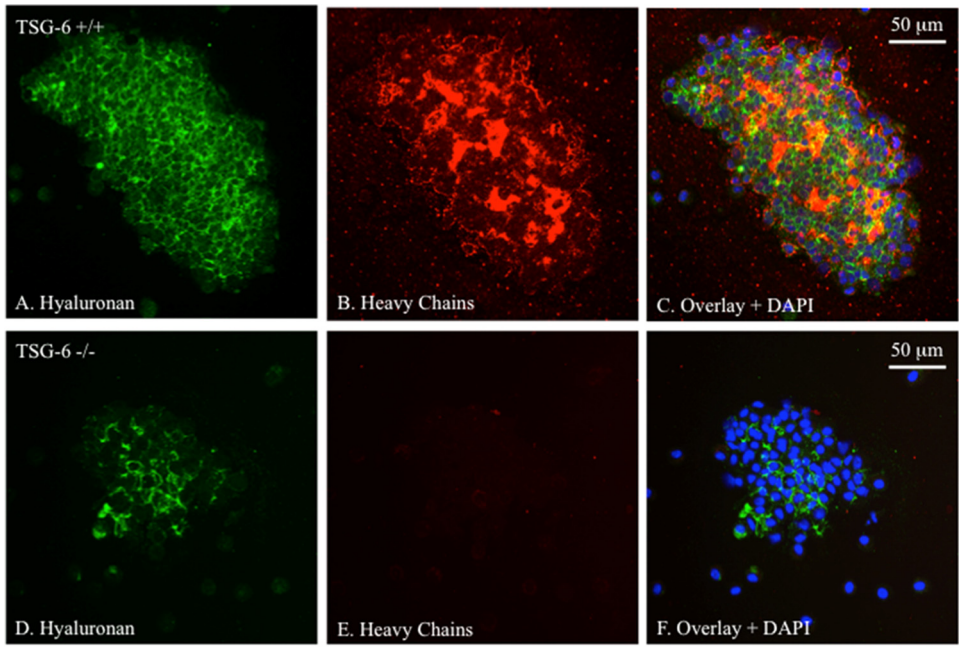

FIGURE 5. **HA rafts in murine BAL require TSG-6 for HC transfer onto HA.** HA rafts are present in BAL from naïve wild type (WT) (*A–C*) and from TSG-6 null mice (*D–F*). HA was stained with a fluorescent conjugate of biotinylated HA-binding protein (*green*), and the presence (*B*) and absence (*E*) of the HC-HA complex was shown by staining with a mixture of HC1, -2, and -3 antibodies (*red*). Leukocytes were stained with DAPI (*blue*) (magnification 20×, *magnification bar* of 50 μm). This was done in replicates of three.

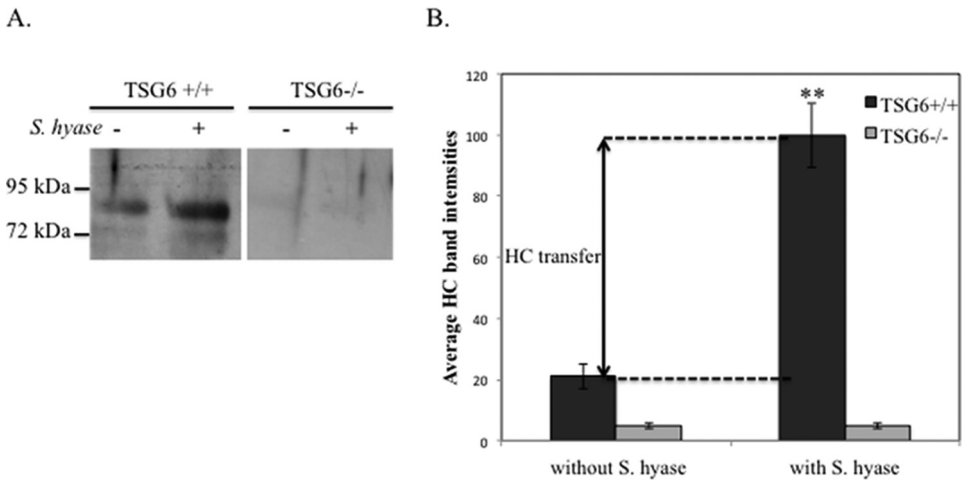

FIGURE 6. **HC-HA complex is found in BAL of naïve WT and not in that of TSG-6 null mice.** *A*, one representative out of three Western blots of BAL from naïve WT and TSG-6 null mice that were incubated without or with *Streptomyces* hyaluronidase (*S. hyase*) and then probed with IαI antibody. *B*, *bar graph* representation of the HCs released by *Streptomyces* hyaluronidase indicates the amount of HC-HA in BAL from naïve WT mice, which is absent in the BAL of TSG-6 null mice.

explanation for leukocyte binding. This adhesion could be due to the involvement of other HA-binding proteins that could be further investigated or, similar to HA cables, HA rafts could bind leukocytes even in the absence of binding proteins such as HCs (38). In fact, the TSG6$^{-/-}$ mice do not exhibit any airway deficiencies or sensitivities under normal conditions unless they are challenged. It has been shown that TSG-6 is essential for the robust increase in pulmonary HA deposition, propagation of acute eosinophilic pulmonary inflammation, and development of airway hyper-responsiveness upon an ovalbumin (OVA/OVA) treatment (19). This emphasizes the role of TSG-6 in the formation of HC-HA complexes and its potential involvement in the HA synthesis during inflammation. Lack of TSG-6 resulted in the lack of HCs in the HA raft found in the BAL of a TSG-6 null mouse. Thus, HC3-HA structure could be required for the cross-linking of HA in a HA raft, which could be a possible explanation for the smaller sized HA raft in the BAL of TSG-6 null mouse compared with WT.

In this study we show that murine AECs can synthesize their own heavy chain donor as pre-IαI complex of HC3 and bikunin (Fig. 7), which was previously reported to be constitutively expressed by human kidney proximal tubular epithelial cells (PTECs) (27). Furthermore, we also show that murine AECs constitutively express TSG-6 (Fig. 8) as do PTECs. However, in their study the production of HC3-bikunin and TSG-6 by human PTECs was investigated in the context of their contribution to the pathogenesis of renal fibrosis. At present it is not clear whether kidney PTECs behave in a similar manner as

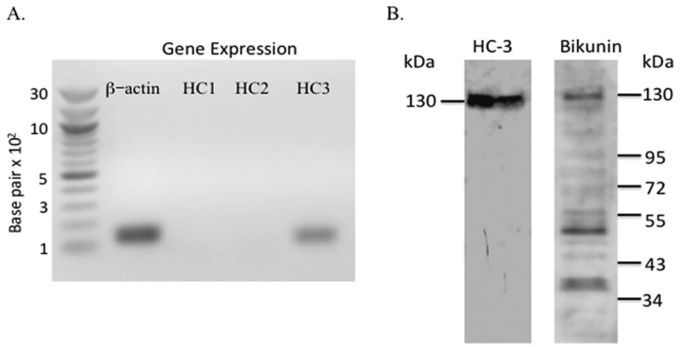

FIGURE 7. **Murine AECs make their own HC3 donor as HC3-bikunin, a member of the PαI/IαI family.** *A*, PCR products produced using primers for the three heavy chain subtypes (HC1–3 as listed in Table 1) were prepared from mRNA isolated from day 10 unstimulated murine AECs and then separated on a 1% agarose gel and stained with 3% ethidium bromide. β-Actin mRNA production was used as the housekeeping gene. One representative gel of three is shown. *B*, Western blots of AEC lysates demonstrate the presence of HC3 protein using a HC3 antibody (*blot on the left*). Western blots with antisera to HC1 and HC2 were negative (data not shown). A biotinylated secondary antibody (1:5000 dilution) was used after the primary antibody application followed by a streptavidin-HRP (1:2000 dilution). The detected band has a molecular mass of 132 kDa, equivalent to that of pre-IαI (with one HC) of the IαI (with two HCs) family. The *blot in the right* also labeled the M_r 132 band when it was probed with an antibody to bikunin, the core protein of IαI, providing evidence for the pre-IαI member of the family that is HC3-bikunin. This was done in replicates of three.

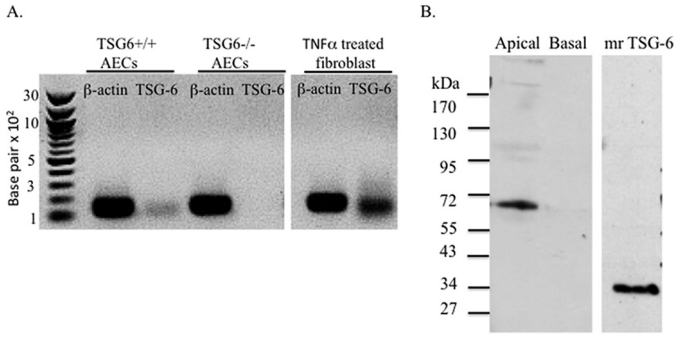

FIGURE 8. **AECs produce TSG-6 apically, which is required for the HC3-HA complex formation.** *A*, TSG-6 expression of day 10 murine AECs is shown by the ethidium bromide-stained PCR product of TSG-6 separated on a 1% agarose gel and by comparison to murine extracts of dermal fibroblast cultures treated with TNFα using β-actin as the housekeeping gene. *B*, Western blot of basal medium and apical washes of day 10 AECs culture were probed with an antibody against TSG-6 that can also detect the murine recombinant TSG-6 (mrTSG-6) used as a positive control. One representative gel of three is shown.

AECs by forming HA rafts. However, both cell types serve as a barrier to the external environment, and HA rafts could be present in both as a means of protection against pathogenic attacks by allowing leukocytes that bind to the HA raft available to perform their function instead of being recruited to the site of inflammation.

The study by Forteza *et al.* (39) also provided evidence that normal human epithelial cell cultures have low levels of mRNA for TSG-6, bikunin, and HC3, which were increased moderately by treatment with TNF-α. The study also showed that epithelia in sections of trachea from smokers showed increased staining for IαI and bikunin compared with normal tracheal sections. This provides evidence that human airway epithelia may also normally make HC3-HA rafts, which are amplified during inflammatory processes such as smoking and asthma.

In summary, this study provides the first description of leukocyte-adhesive HA rafts located in the extracellular space on murine AEC apical surfaces. These HA rafts are identified as HC3-HA complexes that AECs can make in the absence of inflammatory stimuli. Thus, HA rafts perhaps serve as a broad anchor for leukocytes at the airway surface ready to be utilized upon a pathogenic attack or could be an anchor to other proteins and enzymes that might be involved in a host defense mechanism (40). Therefore, this study fills important gaps in our current understanding of murine airway epithelial biology and secretions that relate to human health and disease.

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