Airway Smooth Muscle Cells Synthesize Hyaluronan Cable Structures Independent of Inter-α-inhibitor Heavy Chain Attachment

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The covalent association of inter-α-inhibitor-derived heavy chains (HCs) with hyaluronan was first described in synovial fluid from arthritic patients and later described as a structural and functional component of hyaluronan “cable” structures produced by many different cells and stimuli. HC transfer has been shown to be mediated by the protein product of TSG-6 (tumor necrosis factor-stimulated gene 6). Considering the accumulation of hyaluronan in airways following asthmatic attacks and the subsequent infiltration of leukocytes, we sought to characterize HC substitution of hyaluronan “cables” in primary mouse airway smooth muscle cells (MASM) and primary human airway smooth muscle cells (HASM). We found that cells derived from mice lacking TSG-6 had no defect in hyaluronan production or hyaluronan-mediated leukocyte adhesion when treated with the viral mimic poly(I,C). Functional hyaluronan cables were induced by cycloheximide in the confirmed absence of protein synthesis, with or without simultaneous treatment with poly(I,C). We characterized the species specificity of the antibody other investigators used to describe the HC-hyaluronan complex of hyaluronan cables and found minimal affinity to bovine-derived HCs in contrast to HCs from mouse and human sera. Thus, we cultured MASM and HASM cells in serum from these three sources and analyzed hyaluronan extracts for HCs and other hyaluronan-binding proteins, using parallel cumulus cell-oocyte complex (COC) extracts as positive controls. We conclude that, if hyaluronan cables derived from MASM and HASM cells are substituted with HCs, the amount of substitution is significantly below the limit of detection when compared with COC extracts of similar hyaluronan mass.

This complex (HC-HA) has been found in the sera from patients with rheumatoid arthritis (3), osteoarthritis (3), ovarian cancer (4), cervical ripening (5), and chronic liver disease caused by hepatitis viral infection (6). It has also been found in the bronchial secretions of asthmatics (7) and is an important component of the cumulus cell-oocyte complex in the ovary where it cross-links the hyaluronan matrix and is necessary for female fertility (8–11). HC-HA has been shown to enhance the CD44-mediated binding of leukocytes to hyaluronan isolated from synovial fluid (12) and has been implicated in hyaluronan “cable” structures synthesized by colon mucosal smooth muscle cells (13) and renal proximal tubular epithelial cells in response to the viral mimic poly(I,C) and other stimuli (14).

Iel is primarily a serum macromolecule, synthesized by hepatocytes in the liver (15). It is composed of the following three polypeptides: bikunin (16 kDa) and two HCs (~83 kDa each) (16). The two HCs (HC1 and HC2) are covalently attached to the single chondroitin sulfate glycosaminoglycan of bikunin. Pre-Iel, which consists of a single HC (HC3) attached to the chondroitin sulfate chain, is also formed.

TSG-6 (also known as tumor necrosis factor-induced protein 6) is a 35-kDa protein that is synthesized and secreted by many cells after treatment with tumor necrosis factor and interleukin 1 (17). TSG-6 binds to hyaluronan via its link module and has also been shown to form a complex with both HCs of Iel (8). Furthermore, it is known to catalyze the transfer of HCs from their ester linkage to 6-OH of chondroitin sulfate GalNAc residues on Iel to the 6-OH of GlcNAc residues in hyaluronan (9, 10, 18). Elevated levels of TSG-6 have been observed in asthmatic bronchoalveolar lavage fluid (7), the airway epithelium and secretions of smokers (7), and in infarcted regions following stroke (19). Although there is reason to suggest that other enzymes exist (14, 20), TSG-6 is the only documented enzyme known to catalyze the transfer of HCs from Iel onto hyaluronan.

Hyaluronan “cables” were first observed following viral infection or poly(I,C) treatment of human intestinal mucosal smooth muscle cells (21). These unique strand-like structures polynosinic acid:polycytidylic acid; CHX, cycloheximide; TUN, tunicamycin; COC, cumulus cell-oocyte complex; Iel, inter-α-inhibitor; AbH, polyclonal antibody against human derived inter-α-inhibitor; AbM, polyclonal antibody against mouse heavy chains of inter-α-inhibitor; HC-HA, heavy chain-hyaluronan complex; TSG-6, tumor necrosis factor-stimulated gene 6; HBSS, Hank’s balanced salt solution; FACE, fluorophore-assisted carbodihydrate electrophoresis; PBS, fetal bovine serum; PBS, phosphate-buffered saline.

Since its first description from cultured fibroblasts 15 years ago (1), serum-derived hyaluronan-associated protein has been well characterized as the covalent association of heavy chains (HCs)2 from inter-α-inhibitor (Iel) with hyaluronan (HA) (2).

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2 The abbreviations used are: HC, heavy chain; MASM, mouse airway smooth muscle cells; HASM, human airway smooth muscle cells; poly(I,C) or PIC, polyinosinic acid:polycytidylic acid; CHX, cycloheximide; TUN, tunicamycin; COC, cumulus cell-oocyte complex; Iel, inter-α-inhibitor; AbH, polyclonal antibody against human derived inter-α-inhibitor; AbM, polyclonal antibody against mouse heavy chains of inter-α-inhibitor; HC-HA, heavy chain-hyaluronan complex; TSG-6, tumor necrosis factor-stimulated gene 6; HBSS, Hank’s balanced salt solution; FACE, fluorophore-assisted carbodihydrate electrophoresis; PBS, fetal bovine serum; PBS, phosphate-buffered saline.
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resembled multiple coalescing threads of hyaluronan and were shown to be responsible for the CD44-mediated adhesion of monocellular leukocytes (13). Since then, several other stimulants have been shown to induce hyaluronan cable formation. These include tunicamycin (22), cycloheximide (22), dextran sulfate (22), high glucose (23), BMP-7 (24), and α₁-adrenergic receptor stimulation (25). Hyaluronan cables have been shown to be produced by the following cells: primary human colon smooth muscle cells (21), primary human aortic smooth muscle cells (22), an immortalized human proximal tubular epithelial cell line (24), primary rat mesangial cells (23), and an immortalized rat fibroblast cell line (25).

The association of HCs with hyaluronan cables has been described for human primary colon mucosal smooth muscle cells (13) and an immortalized human proximal tubular epithelial cell line (14). These observations were based on a series of experiments involving immunohistochemical co-localization, inhibition assays, and Western blots using a single polyclonal antibody raised against human-derived 151I. It was concluded that this phenomenon is likely to be essential for the formation, structure, and the leukocyte-adhesive properties of hyaluronan cables (20).

Considering the discovery of the HA-HC complex in asthmatic airway secretions (7), we sought to characterize the HC association with hyaluronan cables derived from primary mouse airway smooth muscle cells (MASMs) and primary human airway smooth muscle cells (HASMs). Through a series of experiments employing antibody-based, biochemical and transgenic methods, we conclude that if HCs are associated with the hyaluronan cables of MASMs and HASMs cells, the amount of substitution is significantly below the limits of detection in relationship to the number of HCs associated with hyaluronan in cumulus cell-oocyte complexes (COCs). Thus, we conclude that hyaluronan cable structures synthesized by MASMs and HASMs cells mediate hyaluronan-based leukocyte adhesion independent of HC attachment and suggest alternative models to explain the properties of this unique hyaluronan structure.

EXPERIMENTAL PROCEDURES

Animals and Animal Care—21-Day-old female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) and housed under conditions of constant temperature with 12-h light/dark cycles. Food and water were available ad libitum. The mice were sacrificed by administering Nembutal (Ovation Pharmaceuticals, Deerfield, IL) at 0.125 mg/g of mouse weight. All protocols with the animals were approved by the Institutional Animal Care and Use Committee. The generation and use of TSG-6 null mice have been described previously (10).

Primary Cell Culture—MASM cells were prepared in the same way as described in the accompanying article (30). HASM were isolated by digesting small bronchial pieces in type II collagenase (LS004174, Worthington) overnight at 37 °C with shaking (120–140 rpm). The supernatant was strained (100 μm, 352340; BD Biosciences) and the filtrate centrifuged (300 × g for 5 min), and the cells were plated (4–5 plates per bilateral transplant). All subsequent culturing was identical to that used for the MASM cells.

Experimental Culture—MASM cells were split 1:4 from passage 1 into 24-well plates for all of the assays in this report. Polyinosinic-polycytidylic acid (poly(I,C)) (P0913, Sigma), cycloheximide (C4859, Sigma), or tunicamycin (T7765, Sigma) were applied to the cells 2 days after splitting. Optimum doses for poly(I,C), cycloheximide, and tunicamycin were 10, 500, and 5 μg/ml, respectively. Treatment duration was 18 h. Treatment volume was 0.5 ml per well. The cells were routinely cultured in Dulbecco’s modified Eagle’s medium/F-12 with 10% FBS, but the FBS content was dropped to 5% for the treatments to facilitate hyaluronan analyses in the conditioned media. Some experiments required 5% mouse serum (S7273, Sigma) or 5% human AB serum (BP2525, Fisher). The mouse and human sera were heat-inactivated at 56 °C for 30 min, and subsequently 0.2 μm filtered.

Leukocyte Adhesion Assay—This procedure is described in the accompanying article (30) and was not altered in this study. Briefly, leukocytes (U937) were labeled with sodium chromate-51 and applied to MASM and HASM cells at 4 °C for 30 min. Unbound leukocytes were gently washed away, and leukocytes bound to hyaluronan were determined by digestion with Streptomyces hyaluronidase. The number of leukocytes remaining bound to the smooth muscle cells was determined by counting a portion of cells (extracted with 1% Triton X-100) on a scintillation counter.

Preparation and Quantification of Hyaluronan for Fluorescent Derivatization with 2-Aminoacridone—This procedure for fluorophore-assisted carbohydrate electrophoresis (FACE) has been described previously (26) and is identical to the method described in the accompanying article (30). Briefly, it involved the purification of hyaluronan via proteinase K digestion and ethanol precipitation. The samples were digested with hyaluronidase SD, which digests hyaluronan into individual disaccharides. These disaccharides were labeled with a fluorophore (2-aminoacridone) and analyzed on a polyacrylamide gel. The hyaluronan band was quantified using imaging software, and the results were presented in this paper in graphical format. This analysis permits a direct measurement of hyaluronan mass.

Immunohistochemistry—MASM and HASM cells were washed three times in Hanks’ balanced salt solution (HBSS) followed by fixation in 4% paraformaldehyde at room temperature for 30 min. The cells were rinsed with HBSS three times and permeabilized with 0.1% Triton X-100 in pre-cooled HBSS at 4 °C for 5 min. A biotinylated hyaluronan-binding protein (product 385911, EMD Chemicals, Gibbstown, NJ) was applied at 1:100 dilution (5 μg/ml) in HBSS containing 1% bovine serum albumin (BP1605-100, Fisher) with either a polyclonal rabbit antibody against I151 (product 385911, EMD Chemicals, Gibbstown, NJ) or the simultaneous incubation of two goat polyclonal HC antibodies (raised against the mouse antigen) at 1:50 (sc-33944 and sc-21978, Santa Cruz Biotechnology) for 45 min. Following washing (HBSS four times), biotinylated hyaluronan-binding protein was subsequently conjugated to streptavidin, Alexa Fluor® 488 (product S11223, Invitrogen) at 1:50 simultaneously with Alexa Fluor® 594.
anti-goat at 1:200 (product A11058, Invitrogen) or Cy3 anti-rabbit at 1:200 (711-165-152, Jackson ImmunoResearch, West Grove, PA).

Isolation of Cumulus Cell–Oocyte Complexes—This procedure has been described previously (8). Briefly, 21-day-old female mice (Charles River Laboratories) were primed by intraperitoneal injection of 5 units of pregnant mare serum gonadotropin (Sigma) in 0.1 ml of phosphate-buffered saline, pH 7.4. COC expansion was induced by injecting 5 units of human chorionic gonadotropin (Sigma) at 46 h after pregnant mare serum gonadotropin priming. Mice were sacrificed 13 h after human chorionic gonadotropin injection, and ovulated cumulus–oocyte complexes were collected from the oviducts.

Hyaluronan Cable Extracts—MASM and HASM cells were treated with (or without) 10 μg/ml poly(I,C) in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 5% fetal bovine, mouse, or human sera. Subsequently, the cells were washed four times at room temperature with PBS followed by the application of 100 μl of 2.86 turbidity reducing units/ml Streptomyces hyaluronidase (100740, Seikagaku, East Falmouth, MA) to each well of a 24-well plate for 10 min. Afterward, the extracts were transferred to a 0.2-ml tube and incubated for an additional 50 min at 37 °C to ensure complete digestion of the extracts. Approximately 30 μl of these undigested extracts were analyzed by Western blot. As a positive control, 50 COCs were digested in 20 μl of the hyaluronidase solution for 10 min and centrifuged at 100 × g for 5 min, transferring the supernatant to a separate tube for further digestion at 37 °C for an additional 50 min. The COC extract was diluted to 500 μl with PBS. Approximately 30 μl (~3 COCs) of these undigested extracts were analyzed by Western blot. In parallel cultures, the hyaluronan content of MASM cells, HASM cells, and COCs were determined by FACE analysis for hyaluronan quantification according to the same cellular proportions described above for the hyaluronidase extracts. This was done to confirm that each of these three populations contained similar hyaluronan concentrations for direct and unbiased comparison.

Western Blot—At the time of harvest, MASM and HASM conditioned media were removed, and the cells were washed four times in PBS. Mammalian protein extraction reagent (M-PER®, 78501, Pierce) containing protease inhibitor mixture (Halt™, 78415, Pierce) containing protease inhibitor mixture (Halt™, 78415, Pierce) was added (60 μl) to each well of a 24-well plate and incubated for 10 min at room temperature. The protein extraction was transferred to a 0.2-ml tube and heated at 100 °C for 10 min with a reducing agent, according to the manufacturer’s recommendation (NP0009, Invitrogen). Approximately 30 μl of the original cell extract was applied to each well of a 10-well SDS-polyacrylamide gel (NP0335BOX, Invitrogen) and blotted to nitrocellulose (926-31090, Li-Cor, Lincoln, NE). The blot was blocked for 1 h with 10 ml of Li-Cor blocking buffer (927-40000, Li-Cor) and probed with antibodies against Iol at 1:1000 (A0301, Dako Cytomation), or the simultaneous incubation of two HC antibodies at 1:200 (sc-33944 and sc-21978, Santa Cruz Biotechnology), or two versican antibodies at 1:1000 (AB1032 and AB1033, Billerica, MA) in the blocking buffer with 0.1% Tween 20 for 1 h. Where appropriate, an antibody against β-actin was applied simultaneously at 1:2,000 as a loading control (A5441, Sigma). The blots were washed five times in PBS with 0.1% Tween 20 and simultaneously probed with IRDYE secondary antibodies (926-32211, 926-32222, and 926-32214, Li-Cor) at 1:15,000 dilution in blocking buffer with 0.1% Tween 20 and 0.01% lauryl sulfate for 45 min. The blots were washed as before and imaged on an Odyssey™ infrared imaging system (Li-Cor).

Statistics and Data Analysis—Throughout this study, all error bars represent standard deviation. p values were determined using the Student’s t test of unpaired data with equal variance (KaleidaGraph, version 3.6, Synergy Software, Reading, PA).

RESULTS

Immunocolocalization of Inter-α-inhibitor Heavy Chain Antibodies with Hyaluronan Cables—In the original report describing hyaluronan cables, a polyclonal antibody raised against Iol isolated from human serum (A0301, Dako Cytomation Denmark; at one time distributed by Novoceastra, Newcastle, UK, as described in Refs. 13, 14)) was shown to co-localize with hyaluronan cables on human colon mucosal smooth muscle cells via immunohistochemistry (13). This observation has also been described by other investigators for an immortalized human proximal tubular epithelial cell line (14). We confirm this observation in smooth muscle cells derived from human airways (Fig. 1, A–C). In contrast, this striking co-localization of the Iol antibody with hyaluronan from HASM was totally absent in smooth muscle cells derived from the MASM (Fig. 1, D–F). We also applied a second pair of polyclonal antibodies raised against mouse HCs and -2 of Iol (sc-33944 and sc-21978, Santa Cruz Biotechnology) to the HASM cells (Fig. 1, G–I) and MASM cells (Fig. 1, J–L). This antibody failed to demonstrate co-localization of HCs with hyaluronan from both the HASM and MASM cells. These observations raise questions regarding antibody specificity, which will be described later in Figs. 4, 5, 7, and 8. To simplify the remainder of this study, we will refer to the Iol antibody as “AbH” (“H” for human antigen) and the two HC antibodies as “AbM” (“M” for mouse antigen).

TSG-6 Is Not Necessary for Hyaluronan Cable Formation and U937 Cell Adhesion—TSG-6 remains the only documented enzyme capable of the covalent transfer of Iol-derived HCs to hyaluronan (9, 10, 18), although there is evidence that other enzymes with similar function may exist (14, 20). We compared MASM cells derived from TSG-6−/− and TSG-6+/+ mice (Fig. 2) to determine whether this enzyme was involved in hyaluronan cable structure and function. Cultures were treated without or with PIC or with TUN. MASM cells from null mice (white bars) and wild-type mice (gray bars) showed no significant difference (p > 0.1 for TUN and p > 0.9 for PIC) in the number of U937 cells bound to hyaluronan cable structures in a standard leukocyte adhesion assay (Fig. 2, A and B). Furthermore, the amounts of hyaluronan retained in the cell layers (Fig. 2C) and present in the media (Fig. 2D) were also independent of TSG-6 (p > 0.1 for both TUN and PIC). These data show that TSG-6 is not necessary for hyaluronan cable formation nor for the adhesion of leukocytes. It is also consistent with the data in Fig. 1, D–F and J–L, which imply that HCs are absent from hyaluronan in MASM cells treated with poly(I,C).
Protein Synthesis Is Not Necessary for Hyaluronan Cable Formation and U937 Cell Adhesion—Cycloheximide (CHX) has been shown previously in aortic smooth muscle cells to induce the synthesis of hyaluronan cables that promote leukocyte adhesion (22). We confirmed this observation in MASM cells, extending it to include a dose response (Fig. 3A and supplemental Fig. 1A and B) and an [3H]leucine assay to confirm the cessation of protein synthesis (supplemental Fig. 1B). CHX stimulated significant (p < 0.0002) hyaluronan-mediated leukocyte adhesion (Fig. 3A) despite only a modest increase in hyaluronan accumulation (supplemental Fig. 1A; p < 0.03). Hyaluronan secretion into the medium was minimal and not affected by CHX (data not shown). Surprisingly, hyaluronan-mediated leukocyte adhesion (Fig. 3A) increased linearly from 10 to 500 μg/ml even though protein synthesis was shown to be absent at 25 μg/ml (supplemental Fig. 1B). This implies that the increase in hyaluronan-mediated leukocyte adhesion at CHX concentrations above 25 μg/ml was not related to further suppression of protein synthesis but perhaps to an unknown toxin in the CHX preparation. Alternatively, it is possible that CHX has an unknown function, independent of the inhibition of protein synthesis, which could induce hyaluronan-mediated leukocyte adhesion. Regardless, the observation that MASM cells promote significant (p < 0.0002) hyaluronan-mediated leukocyte adhesion in the absence of detectable protein synthesis ([3H]leucine incorporation) implies the absence of cell-derived hyaluronan-binding proteins in the hyaluronan cables and/or the lack of the need to up-regulate enzymes (such as TSG-6) to facilitate HC transfer from serum-derived Iα.

Viral Mimic Poly(I,C) and Bacterial Toxin Cycloheximide Induce Hyaluronan-mediated U937 Cell Adhesion by Different Pathways—Because the cessation of protein synthesis induced hyaluronan-mediated leukocyte adhesion by MASM cells, we
tested whether the cessation of protein synthesis would affect hyaluronan cable production and leukocyte adhesion to MASM cells stimulated with poly(I,C). Furthermore, we wanted to see if the hyaluronan response by viral stimulation was additive or competitive with the hyaluronan response induced by the cessation of protein synthesis. Plateau concentrations of 10 μg/ml poly(I,C) and 500 μg/ml CHX induce maximum hyaluronan-mediated leukocyte adhesion to MASM cells, whereas 0.1 μg/ml poly(I,C) and 25 μg/ml CHX are concentrations that induce hyaluronan-mediated leukocyte adhesion at ~50% of the plateau (for poly(I,C) dose response, see the accompanying article (30)). We found that the addition of poly(I,C) and CHX at their mid-plateau doses produced hyaluronan-mediated leukocyte adhesion that was nearly additive (Fig. 3B, gray bar, PIC+CHX; a’ + b’ = c’). Addition of poly(I,C) and CHX at their plateau doses did not result in the additive effect for leukocyte adhesion (Fig. 3B, white bar, PIC+CHX; a + b ≠ c). Interestingly, CHX significantly suppressed (p < 0.0001) poly(I,C)-induced hyaluronan production by MASM cells at the plateau dose, despite its failure to suppress hyaluronan-mediated leukocyte adhesion at the same dose (Fig. 3D, white bars, PIC+CHX versus PIC; a + b ≠ c).

These data imply that poly(I,C) and CHX induce hyaluronan synthesis and mediate leukocyte adhesion via distinct pathways. More importantly, because the cessation of protein synthesis does not prevent poly(I,C)-induced hyaluronan-mediated leukocyte adhesion, hyaluronan-binding proteins are less likely to be involved in the formation, structure, and function of hyaluronan cables.

It is interesting that CHX induces hyaluronan-mediated leukocyte adhesion at levels similar to poly(I,C) induction, whereas hyaluronan production is ~3-fold less (also see our accompanying article (30) for more information about poly(I,C)). Additionally, although the amount of hyaluronan produced by CHX is similar to the amount induced by tunicamycin, the amount of hyaluronan-mediated leukocyte adhesion is ~2-fold more (see our accompanying article (30) for more information about tunicamycin). In other words, CHX induces hyaluronan-mediated leukocyte adhesion at levels comparable with treatment with poly(I,C), although only producing hyaluronan at the lower levels observed with tunicamycin. Although we observed an additive effect of hyaluronan synthesis induced by PIC + tunicamycin at mid-plateau doses (see accompanying article (30)), CHX+PIC at mid-plateau doses was not additive, emphasizing that tunicamycin and CHX operate via distinct pathways.

These data imply that CHX induction of the hyaluronan response is unique among these toxins and that CHX is able to induce a greater number of the MASM cells to produce smaller quantities of hyaluronan cables (similar, but not identical, to tunicamycin). In other words, CHX induces hyaluronan-mediated leukocyte adhesion at levels comparable with treatment with poly(I,C), although only producing hyaluronan at the lower levels observed with tunicamycin. Although we observed an additive effect of hyaluronan synthesis induced by PIC + tunicamycin at mid-plateau doses (see accompanying article (30)), CHX+PIC at mid-plateau doses was not additive, emphasizing that tunicamycin and CHX operate via distinct pathways.

Species-specific Sera Reactivity of Two Inter-α-inhibitor Heavy Chain Antibodies—The failure of AbH and AbM to co-localize with MASM hyaluronan cables in Fig. 1 could be related to the species specificity of the antibodies. Because Iα1 is abundant in mammalian sera, if HCs were to be transferred to hyaluronan during cable formation, the HC donor would likely
come from the 5% serum in the medium as opposed to the relatively small amount of IλI that the smooth muscle cells might produce (see Fig. 5 for more information regarding the amount of I λI produced by MASM and HASM cells). Fig. 4 shows a Western blot of fetal bovine (lanes 1–3), mouse (lanes 4–6), and human (lanes 7–9) sera in which the HCs of IλI were released by digestion with chondroitinase (lanes 2, 5, and 8) or exposure to mild alkaline conditions (lanes 3, 6, and 9) that hydrolyze the ester bonds between the HCs and chondroitin sulfate. AbH recognized the ~83-kDa HC band from both mouse and human sera (Fig. 4A, lanes 5 and 6 and 8 and 9, respectively) but barely detected (176-fold less) HCs from fetal bovine serum (lanes 2 and 3). AbM only recognized HCs derived from mouse serum (Fig. 4B, lanes 5 and 6). Only AbH showed reactivity with bikunin (Fig. 4A, band 5). The identity of the three other bands (Fig. 4A, bands 1–3) are most likely IλI (band 1) and two HCs attached to a common chondroitin sulfate chain (band 2) and pre-IλI (band 3). These data show that AbH has minimal affinity for bovine HCs and much stronger affinity for mouse and human HCs. AbM is only useful to detect mouse HCs. Because it was clear that neither of these antibodies were suitable for detecting the bovine HC antigen (at least via Western blot), the co-localization of AbH with HASM hyaluronan cables in Fig. 1, A–C, is questionable, implying the association of bikunin or intact IλI with the cables. Furthermore, the failure of either AbH or AbM to co-localize with MASM hyaluronan cables in Fig. 1 could simply be because neither antibody reacts well with the bovine antigen from the fetal bovine serum in the medium.

Undetectable Levels of Inter-α-Inhibitor Produced by Murine and Human Airway Smooth Muscle Cells—In Fig. 1, both AbH and AbM stained the HASM and MASM cells, regardless of hyaluronan co-localization, although it is not clear whether this staining was intra- or extracellular. To investigate the apparent cell-associated staining of the HASM and MASM cells, we prepared whole cell, detergent-based extracts of these cells, which were treated with PIC, CHX, or left untreated. These protein extracts were analyzed via Western blot, and the blots were probed with polyclonal antibodies with immunoreactivity against human-derived IλI (AbH) (green bands in A) or murine-derived heavy chains 1 and 2 of IλI (AbM) (green bands in B), and a monoclonal antibody against β-actin as a loading control (red horizontal bands). Molecular weight standards are shown as a vertical column of red bands.
pre-I\(\alpha\) (band 2) were detectable in both MASM and HASM cells using AbH. Because AbM failed to recognize these bands in the MASM cells (Fig. 5B), the most plausible interpretation is that the bands in Fig. 5A are derived from the fetal bovine serum in the culturing medium and not extracted from the cells themselves, despite thoroughly washing (four times PBS) the cells before protein extraction. It is interesting to note that with both the HASM and MASM cells, the amount of I\(\alpha\) and pre-I\(\alpha\) in the protein extracts is slightly higher for the cells treated with poly(I,C) (Fig. 5A, bands 2 and 5; see under “Discussion” for explanation). This implies specific absorption of I\(\alpha\) from the serum in the culturing medium in poly(I,C)-treated cells, possibly mediated by hyaluronan. AbM detected only one band (Fig. 5B, band 3), but the molecular weight of this band (~70 kDa) cannot be easily interpreted as any of the I\(\alpha\) subunits. These results imply that neither HASM nor MASM cells produce their own I\(\alpha\). Thus, they could not be a source for HC transfer to hyaluronan. Furthermore, these results raise doubts regarding the cell-associated I\(\alpha\) staining of Fig. 1.

Hyaluronan Cable Formation and U937 Cell Leukocyte Adhesion to Murine and Human Airway Smooth Muscle Cells Cultured in Different Sera—Because neither MASM nor HASM cells are potential sources for HC transfer to hyaluronan (Fig. 5), if such a process were to occur, it would require a serum donor. Furthermore, it was not known if HC transfer to hyaluronan required the MASM or HASM cells to be cultured in their species-specific serum for this process to occur. Thus, we cultured MASM and HASM cells in 5% fetal bovine, mouse, or human serum during an 18-h poly(I,C) treatment (10 \(\mu\)g/ml) and compared these treatment groups using a standard leukocyte adhesion assay (Fig. 6, A and B) and FACE analysis to measure the hyaluronan content (Fig. 6, C and D). Neither fetal bovine, mouse, nor human sera induced any significant \(p > 0.2\) for all sera) differences in the poly(I,C)-induced leukocyte adhesion for the MASM cells (Fig. 6A, white bars, PIC), although there was a slight (~17%) increase when comparing HASM cells cultured in fetal bovine and human sera with those cultured in mouse sera \(p = 0.002\); Fig. 6B, white bars, PIC). Furthermore, no significant differences were observed in poly(I,C)-induced hyaluronan production (Fig. 6, C and D) by MASM \(p > 0.07\) for all sera) and HASM \(p > 0.1\) for all sera). We were surprised to see the relatively high background of leukocyte adhesion in untreated HASM cells (Fig. 6B), despite the clear hyaluronan-mediated leukocyte binding with PIC treatment (compare white and gray bars). But this observation was also observed three times and in two other patients (data not shown). No hyaluronan cables were observed in untreated HASM cells (data not shown), confirming a non-hyaluronan-mediated binding mechanism for untreated HASM cells, which is exchanged for a hyaluronan-mediated binding following poly(I,C) treatment. Regardless, these results show that both MASM and HASM cells induce significant hyaluronan-mediated leukocyte binding, suggestive of hyaluronan cable formation, and that all sera supported poly(I,C)-induced adhesion to the same extent.

Hyaluronidase Extracts of Airway Smooth Muscle Cell Hyaluronan Cable Structures Fail to Yield Heavy Chains—In parallel cultures from those presented in Fig. 6, we prepared Streptomyces hyaluronidase after binding. Hyaluronan content of MASM (C) and HASM (D) cell layers was determined by FACE analysis. C and D, fetal bovine, mouse, and human sera are represented by white, gray, and black bars, respectively. Error bars represent standard deviation. \(n = 6\) for the U937 cell adhesion assay (A and B). \(n = 8\) for the FACE analysis (C and D).

FIGURE 6. Hyaluronan cable formation and U937 cell adhesion to murine and human airway smooth muscle cells cultured in different sera. Pre-confluent MASM cells (A and C) and HASM cells (B and D) were treated with PIC (10 \(\mu\)g/ml) or untreated (NT) for 18 h in the presence of 3% fetal bovine, mouse, or human serum. Afterward, \(^{51}\)Cr-labeled U937 cells were applied to the MASM cells (A) and HASM cells (B), and hyaluronan-mediated binding was determined by counting (on a scintillation counter) the number of U937 cells remaining after washing with (gray bars) or without (white bars) digestion with Streptomyces hyaluronidase after binding. Hyaluronan content of MASM (C) and HASM (D) cell layers was determined by FACE analysis. C and D, fetal bovine, mouse, and human sera are represented by white, gray, and black bars, respectively. Error bars represent standard deviation. \(n = 6\) for the U937 cell adhesion assay (A and B). \(n = 8\) for the FACE analysis (C and D).
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FIGURE 7. Hyaluronidase extracts of airway smooth muscle cell hyaluronan cable structures fail to yield heavy chains despite doing so for similar cumulus-oophorus extracts. Pre-confluent MASM cells (B and gray bars of A) and HASM cells (C and black bars of A) were treated with PIC (10 μg/ml) or untreated (NT) for 18 h in the presence of 5% fetal bovine, mouse, or human serum (although only 5% fetal bovine serum for A). Hyaluronan content (A) of a defined aliquot of COCs (white bar, represents three COCs) was compared with the hyaluronan contents of defined aliquots of MASM (gray bars, represent the total hyaluronan derived from pre-confluent cells in 1 well of a 24-well plate) and HASM cell layers (black bars, the values are presented as one-fourth the total extract for graphical comparison and represent the total hyaluronan derived from pre-confluent cells in 1 well of a 24-well plate), as determined by FACE analysis, to show that each of the aliquots contained hyaluronan in a similar or greater content as for the COCs. In parallel cultures, COCs, MASM cells (B) and HASM cells (C) were washed four times with room temperature PBS, and hyaluronan-binding proteins were extracted with Streptomyces hyaluronidase digestion in PBS for 10 min at room temperature, transferred to a separate tube, and incubated for a further 50 min at 37 °C to ensure complete digestion. These extracts were analyzed by Western blot, and the blots were probed with a polyclonal antibody with immunoreactivity against human-derived I (AbH) (green bands). Parallel extracts from MASM and HASM cells cultured in 5% mouse serum during hyaluronan cable formation (Fig. 7D), but we found similar results to AbH (Fig. 7, B and C). These results imply that if HC transfer occurs to hyaluronan cables produced by MASM and HASM cells, it is below the limit of detection.

Mouse Airway Smooth Muscle Cells Cultured in Mouse Serum during Poly(I,C)-induced Cable Formation Fail to Demonstrate Heavy Chain Association—In Fig. 1, we showed that hyaluronan cables from MASM cells failed to demonstrate co-localization with either I (AbH) or HC (AbM) antibodies when cultured in fetal bovine serum. As described in Figs. 4, 6, and 7, this failure could be related to the species specificity of the antibodies or the need for the MASM cells to be cultured in their species-specific serum (mouse). In parallel cultures to those presented in Fig. 7, we examined the co-localization of hyaluronan with HCs produced by poly(I,C)-stimulated MASM cells cultured in mouse serum, as determined by confocal microscopy (Fig. 8). Similar to Fig. 1, we were unable to demonstrate HC (AbH) association with hyaluronan cables, even when the MASM cells were cultured in mouse serum instead of fetal bovine serum. We obtained the same result when parallel cultures were probed with AbM (not shown). Thus, although both AbM and AbH have unique species anti-
FIGURE 8. Mouse airway smooth muscle cells cultured in mouse sera during poly(I,C)-induced hyaluronan cable formation fail to demonstrate heavy chain association. Pre-confluent MASM cells were treated with poly(I,C) (10 µg/ml) in the presence of 5% mouse sera for 18 h, fixed in 4% paraformaldehyde for 30 min at room temperature, and co-labeled with a biotinylated hyaluronan-binding protein (green) and an antibody with polyclonal immunoreactivity against I (AbH; red). No co-localization of hyaluronan with I staining was observed by the MASM cells either untreated (A–C) or treated with poly(I,C) (D–F). Parallel cultures of MASM cells probed with AbM gave identical results (data not shown). Cells stained with only 2° antibodies showed minimal background and did not stain the hyaluronan cables (not shown). Magnification is ×40. Magnification bar is 100 µm (C).

FIGURE 9. Hyaluronidase extracts of airway smooth muscle cell hyaluronan cable structures fail to yield versican despite doing so for similar cumulus oophorus extracts. In an experiment similar to Fig. 7, pre-confluent MASM (A) and HASM (B) cells were treated with PIC (10 µg/ml; lanes 5 and 6) or untreated (NT; lanes 2 and 3) for 18 h in the presence of 5% fetal bovine serum. The cells were washed four times with room temperature PBS, and hyaluronan-binding proteins were extracted with Streptococcus hyaluronidase digestion in PBS for 10 min at room temperature, transferred to a separate tube, and incubated for a further 50 min at 37 °C to ensure complete digestion. COC extracts (lanes 1 and 2) were prepared in the same way, as a positive control (described in Fig. 7). These extracts were analyzed by Western blot, and the blots were probed with a polyclonal antibody with immunoreactivity against mouse versican α and β glycosaminoglycan domains (green bands). An aliquot of each sample was digested with chondroitiinase (c′ABC; lanes 2, 4, and 6) to improve antibody binding to versican. Molecular weight standards are shown as red/yellow bands. This experiment was repeated twice using Streptomyces hyaluronidase digests with similar results (not shown).

DISCUSSION

The unexpected result that hyaluronan cable structures did not co-localize with an antibody against I by confocal microscopy in airway smooth muscle cells of mouse origin (Fig. 1), despite doing so for human airway smooth muscle cells (Fig. 1), colon smooth muscle cells (13), and renal proximal tubule cells (14), prompted the series of experiments described in this report. Considering that the only evidence for HC association with hyaluronan cables is derived from various experiments involving this antibody, its species selectivity raised several questions.
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**What Is the Species Specificity for the IαI Antibody (AbH)?**—Because IαI is a multimeric protein composed of three polypeptides, and because AbH is a polyclonal antibody raised against intact IαI, it was important to determine whether AbH showed preferential specificity to any of the polypeptides. We observed in the Western blot of Fig. 4A that AbH has the greatest affinity for IαI of human origin, with the HCs presenting the strongest signal, although bikunin was also clearly recognized. This species specificity is to be expected, because the antibody was raised against the human antigen. Of the other species relevant to this study, AbH recognized IαI of mouse origin giving a strong signal by Western blot, albeit severalfold (3.4 times) less than for the human antigen, presenting its strongest signal on the HC band while lacking any signal for bikunin. Surprisingly, strong signal by Western blot, albeit severalfold (3.4 times) less than for the human antigen, presenting its strongest signal on the HC band while lacking any signal for bikunin. If true, it is more likely that the immunohistochemical co-localization of this antibody with hyaluronan cables, synthesized in the presence of fetal bovine serum, is more likely to represent intact IαI or bikunin than HCs.

Obviously, the specificity of an antibody on a Western blot does not prove its specificity by immunohistochemistry, but this observation, taken with our complete results, raises doubts regarding the ability of AbH to detect HC of bovine origin. Regardless, the implication is that the species of the IαI source is a significant factor in the detection of hyaluronan-associated HCs.

**Does Another Antibody against the Heavy Chains of IαI Give the Same Result?**—Our inability to demonstrate co-localization of AbH with hyaluronan cables of mouse origin prompted the use of an alternative antibody to confirm the results. By Western blot, AbM gave a strong signal for HCs of mouse origin, while completely lacking reactivity with HCs of human and bovine origin. As was expected, AbM failed to recognize bikunin because it was only raised against the HCs. Considering the species specificity of AbM, its failure to co-localize with the MASM hyaluronan cables in Fig. 1 is not surprising because the HC donor would have been from fetal bovine serum. But the failure of AbH and AbM to co-localize with MASM hyaluronan cables that were formed in the presence of mouse serum (Fig. 8) does not support the hypothesis that HCs are associated with MASM-derived hyaluronan cables. More importantly, the failure of AbM to detect HCs from MASM hyaluronidase extracts (Fig. 7D), despite a strong signal from the COCs, is further proof that HCs are not associated with hyaluronan cables derived from airway smooth muscle cells.

**IFHC Transfer Does Occur, What Is the Donor?**—Other investigators have observed that hyaluronan cables could be made in serum-free medium (14). The conclusion from such observations was that the cells were synthesizing their own IαI for HC transfer. Indeed, the cell-associated IαI and HC airway smooth muscle cell immunohistochemistry of Fig. 1 suggests that the cells were making their own IαI, but the Western blots in Fig. 5 fail to support this conclusion. The simplest interpretation from the results of our report is that serum IαI is not necessary in culturing medium because HC transfer is not involved in hyaluronan cable formation by MASM and HASM cells. In a separate study, we showed that serum aids hyaluronan cable formation by MASMs cells (see the accompanying article (30)), but this is most likely because serum provides growth factors that promote the hyaluronan response. It should also be noted in another study that differentiated mouse airway epithelial cells, cultured in serum-free medium, readily produce hyaluronan cables that promote leukocyte adhesion (28). Thus, if HC transfer were to occur in our system, medium serum is the most likely donor.

**How Important Is the Species of the Medium Serum in Relationship to the Species of the Cells Themselves?**—This question is important for two reasons. First, because AbH has only minimal reactivity with HC of bovine origin, and because the most likely IαI donor is the medium serum, cells cultured in fetal bovine serum could give a false negative for HC transfer using AbH. Second, there was the possibility that the MASM- or HASM-associated enzyme(s), such as TSG-6, might require an IαI source specific to their own species, although this is unlikely because it has been shown previously that both endogenous mouse TSG-6 and recombinant human TSG-6 are capable of using bovine IαI as an HC donor in cumulus cell cultures or test tube reactions (10). Thus, for these reasons, we cultured the MASM and HASM cells in fetal bovine, mouse, and human sera during poly(I,C) treatment to induce cable formation (Fig. 6). No significant differences in hyaluronan content or leukocyte adhesion were observed by either species of cells cultured in any of the sera. Additionally, HCs were absent in the hyaluronan cable extracts from each of the sera-mediated cultures, despite their robust presence in parallel extracts from COCs (Fig. 7). These data confirm that our failure to observe HC transfer by the MASM cells in Fig. 1 is not related to their IαI source being of bovine origin, but rather it implies that hyaluronan cable formation by MASM and HASM cells occurs independently of HC transfer.

**Is There Compelling Evidence for Heavy Chain Association with Hyaluronan Cables Using Approaches Not Involving Antibodies?**—Using antibody inhibition assays, others have concluded that TSG-6 is not necessary for hyaluronan cable formation (13, 14). Although other enzymes may exist, TSG-6 is the only documented enzyme known to catalyze HC transfer from IαI to hyaluronan. In Fig. 2, we showed that MASM cells derived from TSG-6 null mice demonstrate no significant differences in hyaluronan content or leukocyte adhesion with wild-type mice treated with or without poly(I,C).

Because MASM cells derived from TSG-6 null mice produce hyaluronan cables in response to poly(I,C), there was no need to determine whether TSG-6 gene expression or protein synthesis was up-regulated during this process. However, assuming that another enzyme might mediate this process, or that other hyaluronan-binding proteins might be involved, protein synthesis would be expected to be involved at some level. The persistence of hyaluronan cable formation in the absence of protein synthesis (Fig. 3) is further evidence that these cables are unlikely to contain HCs or versican (Fig. 9). Additionally, the silver stains of the cable extracts (supplemental Fig. 2) show that if other hyaluronan-binding proteins are associated with hyaluronan cables, they are below the limits of detection.
Although we have raised questions regarding the conclusions of previous results based on AbH to demonstrate HC transfer in hyaluronan cable formation, it should be clear that this antibody is well suited to measure this process if the cells are cultured in medium containing mouse or human sera during cable formation. Nevertheless, we were unable to detect HC association with hyaluronan cable structures using this antiserum. Furthermore, although we cannot conclude that HC transfer is totally absent, the robust signal from COC extracts permits the conclusion that if this transfer truly occurs, then its occurrence is minor in relationship to the COC matrix.

Because it is clear that the HC-hyaluronan association has been observed in sera from patients with a variety of inflammatory states (3–6), synovial fluid from arthritics (12), and bronchial secretions from asthmatics and smokers (7), the interpretation that HCs are not associated with hyaluronan cables in our human and mouse airway smooth muscle in vitro models is not meant to imply that this phenomenon may not occur in vivo during airway inflammation. Furthermore, we are not proposing that HC transfer does not occur in human primary colon mucosal smooth muscle cells (13) or an immortalized human proximal tubular epithelial cell line (14), which has been reported previously. We are simply claiming that the immunohistochemical co-localization of AbH with hyaluronan cables demonstrated for these cells is insufficient evidence to conclude that HC substitution has occurred. More specifically, our observation that AbH almost totally lacks affinity for bovine heavy chains (via Western blot) raises doubts regarding the previous colon smooth muscle cell Western blots of hyaluronidase extracts of hyaluronan cables from cells cultured in fetal bovine serum (13).

There is no question that hyaluronan cables are unique structures with the unique ability to bind leukocytes in contrast to hyaluronan pericellular coats. In other words, there is something unique about the cable structure that permits leukocyte receptors to engage hyaluronan. In the absence of binding proteins, the most compelling alternative to explain this biology is that hyaluronan cables might acquire their unique structure by an undefined, self-associated aggregation of individual hyaluronan strands, distinct from nonassociated strands in pericellular coats. This is plausible because self-association of hyaluronan is known to occur at pH 2.5 and under various ionic conditions (29). Thus, the unique biology of hyaluronan during episodes of inflammation is not limited to HC-hyaluronan association.

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