

AIRWAY SMOOTH MUSCLE CELLS SYNTHESIZE HYALURONAN “CABLE” STRUCTURES INDEPENDENT OF INTER-ALPHA-INHIBITOR HEAVY CHAIN ATTACHMENT

Mark E. Lauer[§], Csaba Fulop[§], Durba Mukhopadhyay[§], Suzy Comhair[¶], Serpil C. Erzurum[¶], Vincent C. Hascall[§].

Running Title: Hyaluronan “Cables” of Airway Smooth Muscle Cells

From the [§]Departments of Biomedical Engineering, and [¶]Pathobiology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44195.

Address Correspondence to: Vincent Hascall, Department of Biomedical Engineering/ ND20, Cleveland Clinic, Cleveland, Ohio 44195; Tel. 216-445-5676; Fax 216-444-9198; E-Mail: hascalv@ccf.org

The covalent association of inter-alpha-inhibitor (I α I) 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 tumor necrosis factor – stimulated gene 6 (TSG-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 to COC extracts of similar hyaluronan mass.

INTRODUCTION

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

I α I is primarily a serum protein, synthesized by hepatocytes in the liver (15). It is composed of the following 3 polypeptides: bikunin (16 kDa) and two HCs (~83 kDa each) (16). The

two HCs (HC1 and HC2) are covalently attached to bikunin's single chondroitin sulfate glycosaminoglycan. Pre-IαI, which consists of a single HC (HC3) attached to the chondroitin sulfate chain, is also formed.

Tumor necrosis factor stimulated gene 6 (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 IαI (8). Furthermore, it is known to catalyze the transfer of HCs from their ester linkage to 6-OH of chondroitin sulfate galNAc residues on IαI 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 IαI 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 resembled multiple coalescing threads of hyaluronan and were shown to be responsible for the CD44-mediated adhesion of mononuclear 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 α_1 -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 immuno-

histochemical co-localization, inhibition assays, and Western blots using a single polyclonal antibody raised against human-derived IαI. 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 MASM and HASM 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 MASM and HASM 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 (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 Cleveland Clinic Institutional Animal Care and Use Committee. The generation and use of TSG-6 null mice has been described previously (10).

Primary Cell Culture – Mouse airway smooth muscle cells (MASM) were prepared in the same way as described in the *companion paper*. Human airway smooth muscle cells (HASM) were isolated by digesting small bronchial pieces in type II collagenase (LS004174,

Worthington Biochemical Corp., Lakewood, NJ) overnight at 37° C with shaking (120-140 rpm). The supernatant was strained (100 µm, 352340, BD Biosciences), the filtrate centrifuged (300 g for 5 min), and the cells 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-Aldrich, St. Louis, MS), cycloheximide (C4859, Sigma-Aldrich) or tunicamycin (T7765, Sigma-Aldrich) 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 DMEM/F12 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-Aldrich, St. Louis, MS) or 5% human AB serum (BP2525, Fisher Scientific, Pittsburg, PA). 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 *companion paper* and was not altered in this study. Briefly, leukocytes (U937) were labeled with sodium ⁵¹chromate 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 *companion paper*. 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 3 times in Hank's balanced salt solution (HBSS) followed by fixation in 4% paraformaldehyde at RT for 30 min. The cells were rinsed with HBSS 3 times, and permeabilized with 0.1% Triton X-100 in pre-cooled HBSS at 4° C for 5 min. A biotinylated hyaluronan binding protein (HABP, product 385911, EMD Chemicals, Gibbstown, NJ) was applied at 1:100 dilution (5 µg/ml) in HBSS containing 1% bovine serum albumin (BP-1605-100, Fisher Scientific) with either a polyclonal rabbit antibody against IαI of human origin at 1:100 (A0301, DAKO Cytomation, Denmark), 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, CA) for 45 min. Following washing (HBSS 4 times) HABP was subsequently conjugated to streptavidin, Alexa Fluor® 488 (product S11223, Invitrogen, Carlsbad, CA) at 1:500 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, Wilmington, MA) were primed by intraperitoneal injection of 5 units of pregnant mares' serum gonadotropin (PMSG, Sigma-Aldrich) in 0.1 ml phosphate-buffered saline, pH 7.4. COC expansion was induced by injecting 5 units of human chorionic gonadotropin (hCG, Sigma-Aldrich) at 46 h after PMSG priming. Mice were sacrificed 13 h after hCG injection,

and ovulated cumulus-cell 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 DMEM/F12 medium supplemented with 5% fetal bovine, mouse or human sera. Subsequently, the cells were washed 4 times with RT PBS followed by the application of 100 µl 2.86 TRU/ml *Streptomyces* hyaluronidase (100740, Seikagaku, East Falmouth, MA) to each well of a 24-well plate for 10 min. Afterwards, the extracts were transferred to a 0.2 ml tube and incubated for an additional 50 min at 37°C to insure complete digestion of the extracts. Approximately 30 µl of these undiluted 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 undiluted 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 3 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 4 times in PBS. Mammalian Protein Extraction Reagent (M-PER®, 78501, Pierce Rockford, IL) containing Protease Inhibitor Cocktail (Halt™, 78415, Pierce) was added (60 µl) to each well of a 24-well plate and incubated for 10 min at RT. 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 manufacturer’s recommendation (NP0009, Invitrogen, Carlsbad, CA). Approximately 30 µl of the original cell extract was applied to each well of a 10-well SDS-PAGE gel (NP0335BOX, Invitrogen, Carlsbad, CA) and blotted to nitrocellulose (926-

31090, Li-Cor, Lincoln, NE). The blot was blocked for 1 h with 10 ml Li-Cor blocking buffer (927-40000, Li-Cor) and probed with antibodies against IαI at 1:1000 (A0301, DAKO Cytomation, Denmark), or the simultaneous incubation of two HC antibodies at 1:200 (sc-33944 and sc-21978, Santa Cruz Biotechnology, CA) 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-Aldrich). The blots were washed 5 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 manuscript, all error bars represent standard deviation. P –values were determined using the Student t – Test of unpaired data with equal variance (Kaleidagraph, v3.6, Synergy Software, Reading, PA).

RESULTS

Immunocolocalization of inter-alpha-inhibitor heavy chain antibodies with hyaluronan “cables” In the original report describing hyaluronan “cables,” a polyclonal antibody raised against inter-alpha-inhibitor (IαI) isolated from human serum (A0301, DAKO Cytomation, Denmark; at one time distributed by Novocastra, Newcastle, UK, as described in (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. 1A – C). In contrast, this striking co-localization of the IαI antibody with hyaluronan from *human* airway smooth muscle cells (HASM) was totally absent

in smooth muscle cells derived from the *mouse* airway (MASM; fig. 1D – F). We also applied a second pair of polyclonal antibodies raised against mouse heavy chains (HCs) 1 and 2 of IaI (sc-33944 and sc-21978, Santa Cruz Biotechnology, CA) to the HASM cells (fig. 1G – I) and MASM cells (fig. 1J – 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 figures 5, 6 and 8, 9. To simplify the remainder of this report, we will refer to the IaI 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. Tumor necrosis factor-stimulated Gene – 6 (TSG-6) remains the only documented enzyme capable of the covalent transfer of IaI 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 *+/+* mice (fig. 2) to determine if this enzyme was involved in hyaluronan “cable” structure and function. Cultures were treated without (NT), or with poly I:C (PIC), or with tunicamycin (TUN). MASM cells from null mice (white bars) and wild type mice (grey bars) showed no significant difference ($p > 0.1$ for TUN and > 0.9 for PIC) in the number of U937 cells bound to hyaluronan “cable” structures in a standard leukocyte adhesion assay (fig. 2A 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 figure 1, panels 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

previously shown 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 supplement 1A) and a ^3H -leucine assay to confirm the cessation of protein synthesis (supplement 1B). CHX stimulated significant ($p < 0.0002$) hyaluronan-mediated leukocyte adhesion (fig. 3A) in spite of only a modest increase in hyaluronan accumulation (supplement 1A; $p < 0.03$). Hyaluronan secretion into the medium was minimal and not affected by CHX (not shown). Surprisingly, hyaluronan-mediated leukocyte adhesion (fig. 3A) increased linearly from 10 to 500 $\mu\text{g/ml}$ even though protein synthesis was shown to be absent at 25 $\mu\text{g/ml}$ (supplement 1B). This implies that the increase in hyaluronan-mediated leukocyte adhesion at CHX concentrations above 25 $\mu\text{g/ml}$ 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 upregulate enzymes (such as TSG-6) to facilitate HC transfer from serum-derived IaI.

The viral mimic poly(I:C) and the bacterial toxin cycloheximide induce hyaluronan mediated U937 cell adhesion by different pathways. Since 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 $\mu\text{g/ml}$ poly(I:C)

and 500 $\mu\text{g/ml}$ CHX induce maximum hyaluronan-mediated leukocyte adhesion to MASM cells, whereas 0.1 $\mu\text{g/ml}$ poly(I:C) and 25 $\mu\text{g/ml}$ CHX are concentrations that induce hyaluronan-mediated leukocyte adhesion $\sim 50\%$ of the plateau (for poly(I:C) dose-response, see *companion paper*). 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, grey 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; $a + b \neq c$). 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 vs. PIC; $a + b \neq c$).

These data imply that poly(I:C) and CHX induce hyaluronan synthesis and mediate leukocyte adhesion via distinct pathways. More importantly, since 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 while hyaluronan production is about ~ 3 fold less (also see our *companion paper* for more information about poly(I:C)). Additionally, while the amount of hyaluronan produced by CHX is similar to the amount induced by tunicamycin, the amount of hyaluronan-mediated leukocyte adhesion is about ~ 2 fold more (see our *companion paper* for more information about tunicamycin). In other words, CHX induces hyaluronan-mediated leukocyte adhesion at levels comparable to treatment with poly(I:C), while only producing hyaluronan at the lower levels observed with tunicamycin. While we observed an additive effect of hyaluronan synthesis induced by PIC + tunicamycin at mid-plateau doses (see *companion paper*), 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 our conclusion regarding the differences between poly(I:C) and tunicamycin).

Species specific sera reactivity of two inter-alpha-inhibitor heavy chain antibodies. The failure of AbH and AbM to colocalize with MASM hyaluronan “cables” in figure 1 could be related to the species specificity of the antibodies. Since I α I 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 I α I that the smooth muscle cells might produce (see fig. 5 for more information regarding the amount of I α I produced by MASM and HASM cells). Figure 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 I α I were released by digestion with chondroitinase (lanes 2, 5, 8) or exposure to mild alkaline conditions (lanes 3, 6, 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, 6 and 8, 9, respectively), but barely detected (176 fold less) HCs from fetal bovine serum (lanes 2, 3). AbM only recognized HCs derived from mouse serum (fig. 4B, lanes 5, 6). Only AbH showed reactivity with bikunin (fig. 4A, band 5). The identity of the 3 other bands (bands 1-3) are most likely I α I (band 1), two HCs attached to a common chondroitin sulfate chain (band 2) and pre-I α I (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. Since 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 figure 1A-C is questionable, implying the association of bikunin or intact I α I with the “cables.” Furthermore, the failure of either AbH or AbM

to colocalize with MASM hyaluronan cables in figure 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-alpha-inhibitor produced by murine and human airway smooth muscle cells. In figure 1, both AbH and AbM stained the HASM and MASM cells, regardless of hyaluronan co-localization, though it is not clear whether this staining was intra- or extracellular. In order 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 poly(I:C) (PIC), cycloheximide (CHX) or left untreated (NT). These protein extracts were analyzed via Western blot, probing the blots with AbH (fig. 5A) and AbM (fig. 5B). I α I (band 1) and pre-I α I (band 2) were detectable in both MASM and HASM cells using AbH. Since AbM failed to recognize these bands in the MASM cells (fig. 5B), the most plausible interpretation is that the bands in figure 5A are derived from the fetal bovine serum in the culturing medium, and not extracted from the cells themselves, despite thoroughly washing (4 times PBS) the cells before protein extraction. It is interesting to note that with both the HASM and MASM cells, the amount of I α I and pre-I α I in the protein extracts is slightly higher for the cells treated with poly(I:C) (lanes 2 and 5; see discussion for explanation). This implies specific absorption of I α I from the serum in the culturing medium in poly(I:C) treated cells, possibly mediated by hyaluronan. AbM detected only one band (band 3), but the molecular weight of this band (~70 kDa) cannot be easily interpreted as any of the I α I subunits. These results imply that neither HASM, nor MASM cells produce their own I α I. Thus, they could not be a source for HC transfer to hyaluronan. Furthermore, these results raise doubts regarding the cell-associated I α I staining of figure 1.

Hyaluronan “cable” formation and U937 cell leukocyte adhesion to murine and human airway smooth muscle cells cultured in different sera. Since 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 μ g/ml) and compared these treatment groups using a standard leukocyte adhesion assay (fig. 6A and B) and FACE analysis to measure the hyaluronan content (fig. 6C 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. 6C 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 (NT) HASM cells (fig. 6B), despite the clear hyaluronan-mediated leukocyte binding with poly(I:C) (PIC) treatment (compare white and grey bars). But this observation was also observed 3 times and in 2 other patients (not shown). No hyaluronan “cables” were observed in untreated HASM cells (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 extracts of the cells, to release any HCs that might be covalently bound to hyaluronan, and analyzed them via Western blot (fig. 7B, C and D). As a positive control of HC

transfer to hyaluronan, we prepared hyaluronidase extracts from cumulus cell - oocyte complexes (COCs) in the identical manner as for the MASM and HASM cells. In order to show that the hyaluronidase extracts of figure 7B, C and D represented digests from similar levels of hyaluronan, we measured the amounts of hyaluronan from the COCs and the extracts from the HASM and MASM cells by FACE analysis for comparison of loading (fig. 7A). This demonstrated that the amount of hyaluronan from the MASM cell extracts (grey bars) was similar to the amount of hyaluronan from the COC extracts (white bar), while HASM cell extracts (black bars) had nearly 4 times the amount of hyaluronan as the COCs. Despite the robust HC band of the COC hyaluronidase extract (lane 1), neither MASM (fig. 7B) nor HASM (fig. 7C) cell hyaluronidase extracts showed the release of HC from hyaluronan "cable" structures when cultured under any of the different sera conditions, as determined by immunoreactivity with AbH. Since AbM only recognizes the mouse HC antigen, we also used this antibody to probe a blot containing hyaluronidase extracts from MASM and HASM cells cultured in 5% mouse serum during hyaluronan "cable" formation (fig. 7D), but found similar results to AbH (fig. 7B 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 figure 1, we showed that hyaluronan "cables" from MASM cells failed to demonstrate colocalization with either I α I (AbH) nor HC (AbM) antibodies when cultured in fetal bovine serum. As described in figure 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 colocalization 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 figure 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 antigenicities, it appears that our failure to demonstrate HC association with hyaluronan "cables" produced by MASM cells is not related to the species antigenicities of the antibodies, nor the need for a species specific HC donor in the MASM cell system.

Hyaluronidase extracts of airway smooth muscle cell hyaluronan "cable" structures fail to yield versican despite doing so for similar cumulus oophorus extracts. It has been previously reported that versican is a hyaluronan binding protein associated with hyaluronan cables (14,27) as determined by immunohistochemistry. Using similar MASM (fig. 9A) and HASM (fig. 9B) *Streptococcus* hyaluronidase extracts from similar cultures as presented in fig. 7, we investigated the presence of versican by Western blot from samples that had been digested with chondroitinase to expose versican epitopes. Versican was clearly present in COC extracts as a smear (lane 1) that resolved to reveal the core protein after chondroitinase digestion (lane 2). If versican was present in the MASM and HASM hyaluronidase extracts (lanes 3-6 of panels A and B, respectively), then it was below the limits of detection, and significantly less than what was observed in COC extracts.

In addition to the Western blots, we also analyzed parallel samples from the hyaluronidase extracts of figure 7 by silver staining SDS-PAGE gels in an attempt to discover any hyaluronan binding proteins associated with poly(I:C) induced hyaluronan "cables" (supplemental 2). In neither of the 3 sera conditions did we observe a band in poly(I:C) treated cultures (PIC) that was different from untreated cultures (NT) for either the MASM or the HASM cell extracts.

DISCUSSION

The unexpected result that hyaluronan "cable" structures did not co-localize with an

antibody against IαI 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.

What is the species specificity for the IαI antibody (AbH)? Since IαI is a multimeric protein composed of 3 polypeptides, and since AbH is a polyclonal antibody raised against intact IαI, it was important to determine if AbH showed preferential specificity to any of the polypeptides. We observed in the Western blot of figure 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, since 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 several fold (3.4x) less than for the human antigen, presenting its strongest signal on the HC band while lacking any signal for bikunin. Surprisingly, although AbH gave a strong signal against IαI and pre-IαI of bovine origin, HC antigenicity was almost non-existent (176x fold less than HCs of human origin). Thus, it is clear that the primary affinity that AbH has for IαI of bovine origin is 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 colocalization of AbH with hyaluronan “cables” of *mouse* origin prompted the use of an alternative antibody to confirm the results. By Western, 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 figure 1 is not surprising since the HC donor would have been from fetal bovine serum. But the failure of AbH and AbM to colocalize 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.

If HC 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 figure 1 suggests that the cells were making their own IαI, but the Western blots in figure 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 MASM cells (see *companion paper*), 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, since AbH has only minimal reactivity with HC of bovine origin, and since 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. Secondly, 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 since it has been shown previously that both endogenous mouse TSG-6 and recombinant human TSG6 are capable of using bovine IαI as a 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 figure 1 is not related to their IαI source being of bovine origin, but rather 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 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 figure 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).

Since MASM cells derived from TSG-6 null mice produced hyaluronan “cables” in response to poly(I:C), there was no need to determine if 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 (supplement 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, its occurrence is minor in relationship to the COC matrix.

Since 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 previously reported. We are simply claiming that the immuno-histochemical colocalization 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 Westerns 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 non-associated strands in pericellular coats. This is plausible since 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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FOOTNOTES

Abbreviations:

Mouse airway smooth muscle cells (MASM); human airway smooth muscle cells (HASM); polyinosinic acid:polycytidylic acid (poly(I:C) or PIC); cycloheximide (CHX); tunicamycin (TUN); cumulus cell – oocyte complex (COC); heavy chain (HC); inter-alpha-inhibitor (IαI); polyclonal antibody against human derived inter-alpha-inhibitor (AbH); polyclonal antibody against mouse heavy chains of inter-alpha-inhibitor (AbM); heavy chain – hyaluronan complex (HC – HA); serum derived hyaluronan associated protein (SHAP); tumor necrosis factor – stimulated gene 6 (TSG-6); vascular cell adhesion molecule 1 (VCAM – 1); Hank’s balanced salt solution (HBSS); biotinylated hyaluronan binding protein (HABP); fluorophore-assisted carbohydrate electrophoresis (FACE).

Acknowledgments:

We would like to acknowledge the valuable advice of Carol A. de la Motte and Alana K. Majors (Department of Pathobiology and Colorectal Surgery, Lerner Research Institute, Cleveland Clinic) throughout this project. We would also like to thank Tibor Glant and Katalin Mikecz (Rush University Medical Center, Chicago, IL) for generously providing the TSG-6 knockout mice. The research in this manuscript was funded by NIH PO1 HL081064 Pathology of Asthma.

FIGURE LEGENDS

Fig. 1 *Immunocolocalization of inter-alpha-inhibitor heavy chain antibodies with hyaluronan “cables.”* Pre-confluent HASM cells (panels A – C and G – I) and MASM cells (panels D – F and J – L) were treated with poly(I:C) (10 µg/ml) in the presence of 5% FBS for 18 h, fixed in 4% paraformaldehyde for 30 min at RT and co-labeled with a biotinylated hyaluronan binding protein (green) and an antibody with polyclonal immunoreactivity against human derived IαI (AbH; red, panels A – F) or mouse derived heavy chains 1 and 2 of IαI (AbM; red, panels G – L). Colocalization of hyaluronan with IαI staining was only observed in HASM cells (panels A – C). Hyaluronan cables were absent from cells not treated with poly(I:C), although cell associated staining for both antibodies was observed (not shown). Cells stained with only 2° antibodies showed minimal background and did not stain the hyaluronan “cables” (not shown). Mag. is 40x. Mag. bar is 100 µm (panel L). This experiment was repeated 2 times with the heavy chain antibody (AbM) and more than 4 times with the IαI antibody (AbH).

Fig. 2 *TSG-6 is not necessary for hyaluronan “cable” formation and U937 cell adhesion.* Pre-confluent MASM cells, derived from wild-type (grey bars) or TSG-6 null (white bars) mice were untreated (NT), treated with tunicamycin (TUN; 5 µg/ml) or treated with poly(I:C) (PIC; 10 µg/ml) for 18 h. Afterward, ⁵¹Cr-labeled U937 cells were applied to the MASM cells and hyaluronan-mediated binding was determined by counting (on a scintillation counter) the number of U937 cells remaining after washing with (panel B) or without (panel A) treatment with *Streptomyces* hyaluronidase after binding. The hyaluronan contents of MASM cell layers (panel C) and conditioned medium (panel D) were determined by FACE analysis. Error bars represent standard deviation. P – values of < 0.1 and 0.9 are labeled with * and ** respectively. N = 4 for the U937 cell adhesion assay (panels A and B). N = 6 for the FACE analysis (panels C and D).

Fig. 3 *The viral mimic poly(I:C) and the bacterial toxin cycloheximide induce hyaluronan mediated U937 cell leukocyte adhesion by different mechanisms.* Pre-confluent MASM cells were cultured in the presence of cycloheximide at 0, 10, 25, 50, 100, 250 and 500 µg/ml for 18 h (panel A). Afterwards, ⁵¹Cr-labeled U937 cells were applied to the MASM cells, and hyaluronan-mediated binding was determined by counting (on a scintillation counter) the number of U937 cells remaining after washing with (grey bars) or without (white bars) digestion with *Streptomyces* hyaluronidase after binding. In a separate study, pre-confluent MASM cells were cultured in the presence of poly(I:C) (PIC) and/or cycloheximide (CHX) at concentrations that gave a maximum plateau (white bars) and mid-plateau (grey bars) response for the U937 cell adhesion assay. These concentrations were 10 and 0.5 µg/ml for poly(I:C) and 500 and 25 µg/ml for cycloheximide, respectively. Afterwards, U937 cells were applied to the MASM cells as described in panel A, with (panel C) or without (panel B) digestion with *Streptomyces* hyaluronidase after binding. Hyaluronan content of MASM cell layers (panel D) was determined by FACE analysis. The values for untreated control cultures are presented as dashed lines (panels B – D). In panel B, $a + b \neq c$ whereas $a' + b' = c'$ (see results for further description). In panel D, $a + b \neq c$ and $a' + b' \neq c'$ (see results for further description). Error bars represent standard deviation. P – values < 0.0002 are labeled with an asterisk. N = 3 for the U937 cell adhesion assay (panels A, B and C). N = 4 for the FACE analysis (panel D).

Fig. 4 *Species specific sera reactivity of two inter-alpha-trypsin inhibitor heavy chain antibodies.* Aliquots of fetal bovine serum (lanes 1-3), mouse serum (lanes 4-6) and human serum (lanes 7-9) were subjected to chondroitinase (c'ABC) or mild alkaline (0.1 M NaOH at RT for 10 min) treatment and analyzed by Western blot. The blots were probed with polyclonal antibodies with immunoreactivity against human derived IαI (AbH) (panel A) or murine derived heavy chains 1 and 2 of IαI (AbM) (panel B). Molecular weight standards are shown as red bands. Sera bands are shown in green. This experiment gave identical results when compared with 2 different lots of fetal bovine serum and human serum (not shown).

Fig. 5 Undetectable levels of inter-alpha-inhibitor produced by murine and human airway smooth muscle cells. Pre-confluent HASM cells (lanes 1-3) and MASM cells (lanes 4-6) were cultured in the presence of poly(I:C) (PIC; 10 µg/ml), cycloheximide (CHX; 500 µg/ml), or untreated (NT) for 18 h. Afterwards, the medium was aspirated, and the cells were rinsed 4 times with PBS followed by a detergent based protein extraction. These protein extracts were analyzed by Western blot, and the blots were probed with polyclonal antibodies with immunoreactivity against human derived IαI (AbH) (green bands in panel A) or murine derived heavy chains 1 and 2 of IαI (AbM) (green bands in panel 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.

Fig. 6 Hyaluronan “cable” formation and U937 cell adhesion to murine and human airway smooth muscle cells cultured in different sera. Pre-confluent MASM cells (panels A and C) and HASM cells (panels B and D) were treated with poly(I:C) (PIC; 10 µg/ml) or untreated (NT) for 18 h in the presence of 5% fetal bovine, mouse or human serum. Afterwards, ⁵¹Cr-labeled U937 cells were applied to the MASM cells (panel A) and HASM cells (panel B), and hyaluronan-mediated binding was determined by counting (on a scintillation counter) the number of U937 cells remaining after washing with (grey bars) or without (white bars) digestion with *Streptomyces* hyaluronidase after binding. Hyaluronan content of MASM (panel C) and HASM (panel D) cell layers was determined by FACE analysis. In panels C and D, fetal bovine, mouse and human serum are represented by white, grey and black bars, respectively. Error bars represent standard deviation. N = 6 for the U937 cell adhesion assay (panels A and B). N = 8 for the FACE analysis (panels C and D).

Fig. 7 Hyaluronidase extracts of airway smooth muscle cell hyaluronan “cable” structures fail to yield heavy chains despite doing so for similar cumulus cell - oophorus extracts. Pre-confluent MASM cells (panel B and grey bars of panel A) and HASM cells (panel C and black bars of panel A) were treated with poly(I:C) (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 panel A). Hyaluronan content (panel A) of a defined aliquot of cumulus cell - oocyte complexes (COCs; white bar; represents 3 COCs) was compared with the hyaluronan contents of defined aliquots of MASM (grey bars; represent the total hyaluronan derived from pre-confluent cells in 1 well of a 24-well plate) and HASM (black bars; the values are presented as ¼ the total extract for graphical comparison and represent the total hyaluronan derived from pre-confluent cells in 1 well of a 24-well plate) cell layers, 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 (panel B) and HASM cells (panel C) were washed 4 times with RT PBS and hyaluronan-binding proteins were extracted with *Streptomyces* hyaluronidase digestion in PBS for 10 min at RT, transferred to a separate tube, and incubated for a further 50 min at 37° C to assure complete digestion. These extracts were analyzed by Western blot, and the blots were probed with a polyclonal antibody with immunoreactivity against human derived IαI (AbH) (green bands). Parallel extracts from MASM and HASM cells cultured in 5% mouse serum were also analyzed by Western blot and probed with AbM (panel D). Molecular weight standards are shown as red bands. Error bars represent standard deviation. N = 2 for the COCs and N = 4 for the MASM and HASM cells of the FACE analysis (panel A). For panels B and C, this experiment was repeated 3 times. The data in panel D were repeated 2x.

Fig. 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 RT and co-labeled with a biotinylated hyaluronan binding protein (green) and an antibody with polyclonal immunoreactivity against IαI (AbH; red). No colocalization of hyaluronan with IαI staining was observed by the MASM cells either untreated (panels A – C) or treated with poly(I:C) (panels D – F). Parallel cultures of MASM cells probed with AbM gave identical results (not shown). Cells stained with

only 2° antibodies showed minimal background and did not stain the hyaluronan “cables” (not shown). Mag. is 40x. Mag. bar is 100 μm (panel C).

Fig. 9 *Hyaluronidase extracts of airway smooth muscle cell hyaluronan “cable” structures fail to yield versican despite doing so for similar cumulus cell - oophorus extracts.* In an experiment similar to figure 7, pre-confluent MASM (panel A) and HASM (panel B) cells were treated with poly(I:C) (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 4 times with RT PBS and hyaluronan-binding proteins were extracted with *Streptococcus* hyaluronidase digestion in PBS for 10 min at RT, transferred to a separate tube, and incubated for a further 50 min at 37° C to assure complete digestion. COC extracts (lanes 1 and 2) were prepared in the same way, as a positive control (described in fig. 8). 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 chondroitinase (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 2x using *Streptomyces* hyaluronidase digests with similar results (not shown).

FIGURE 1

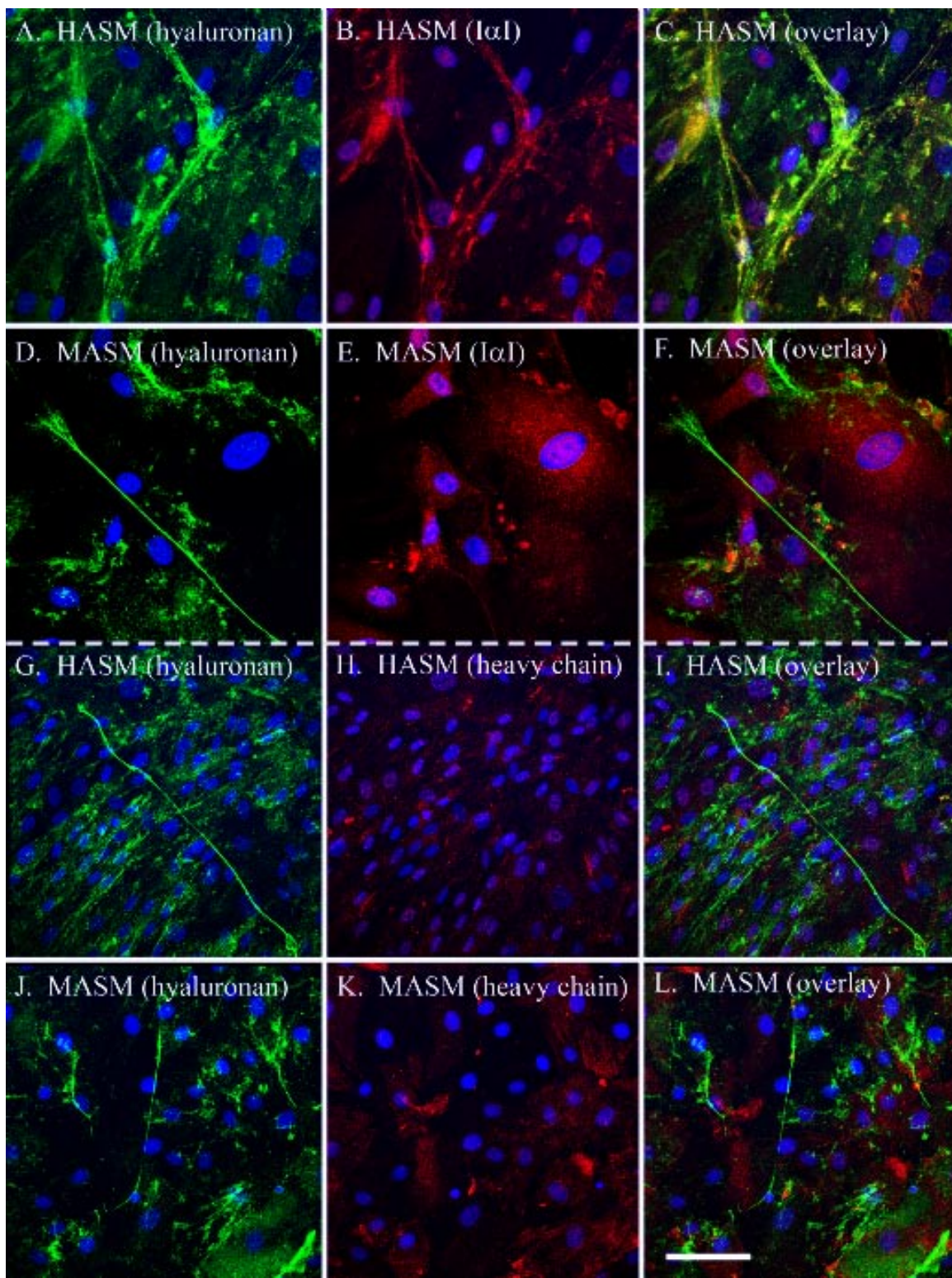


FIGURE 2

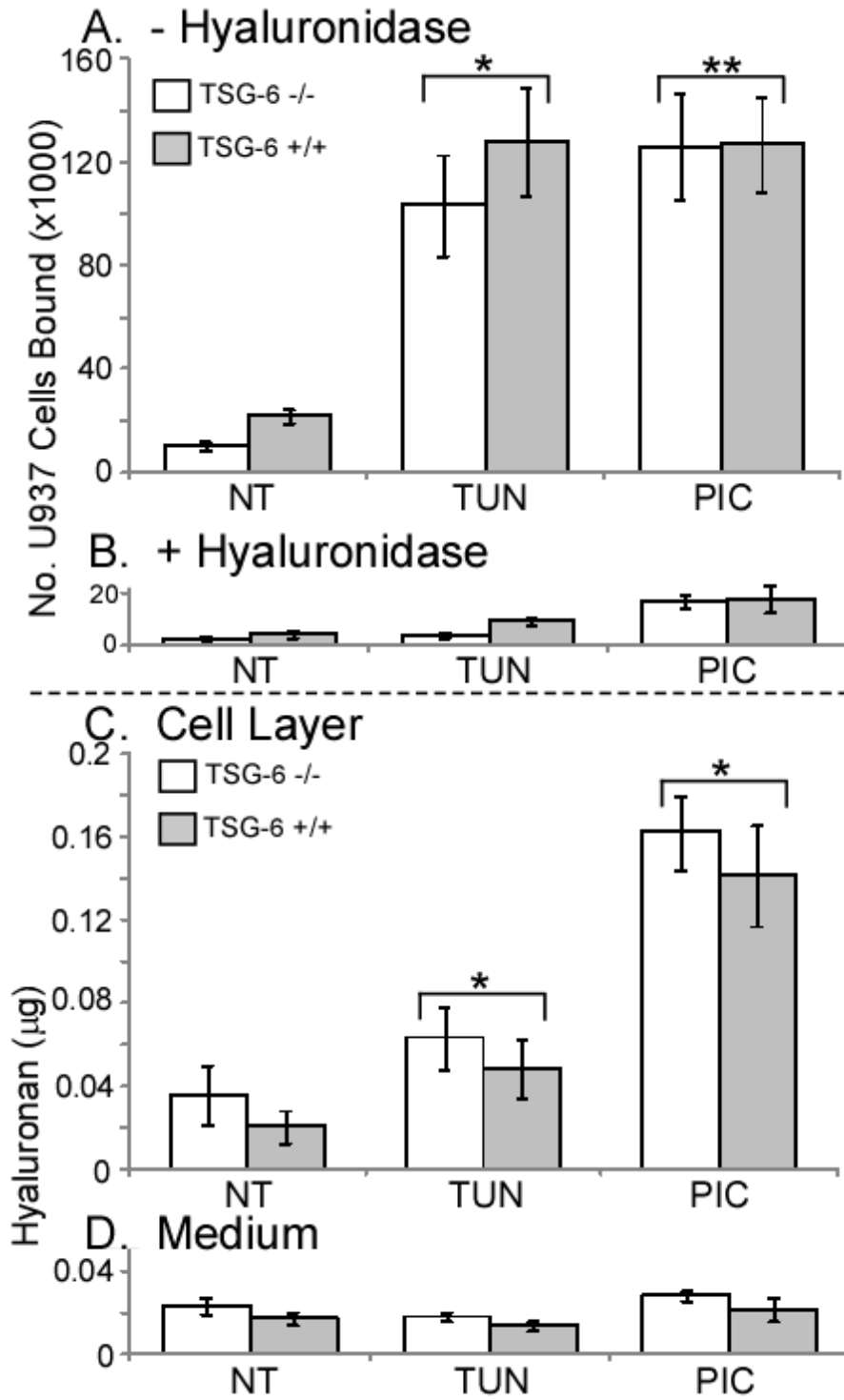


FIGURE 3

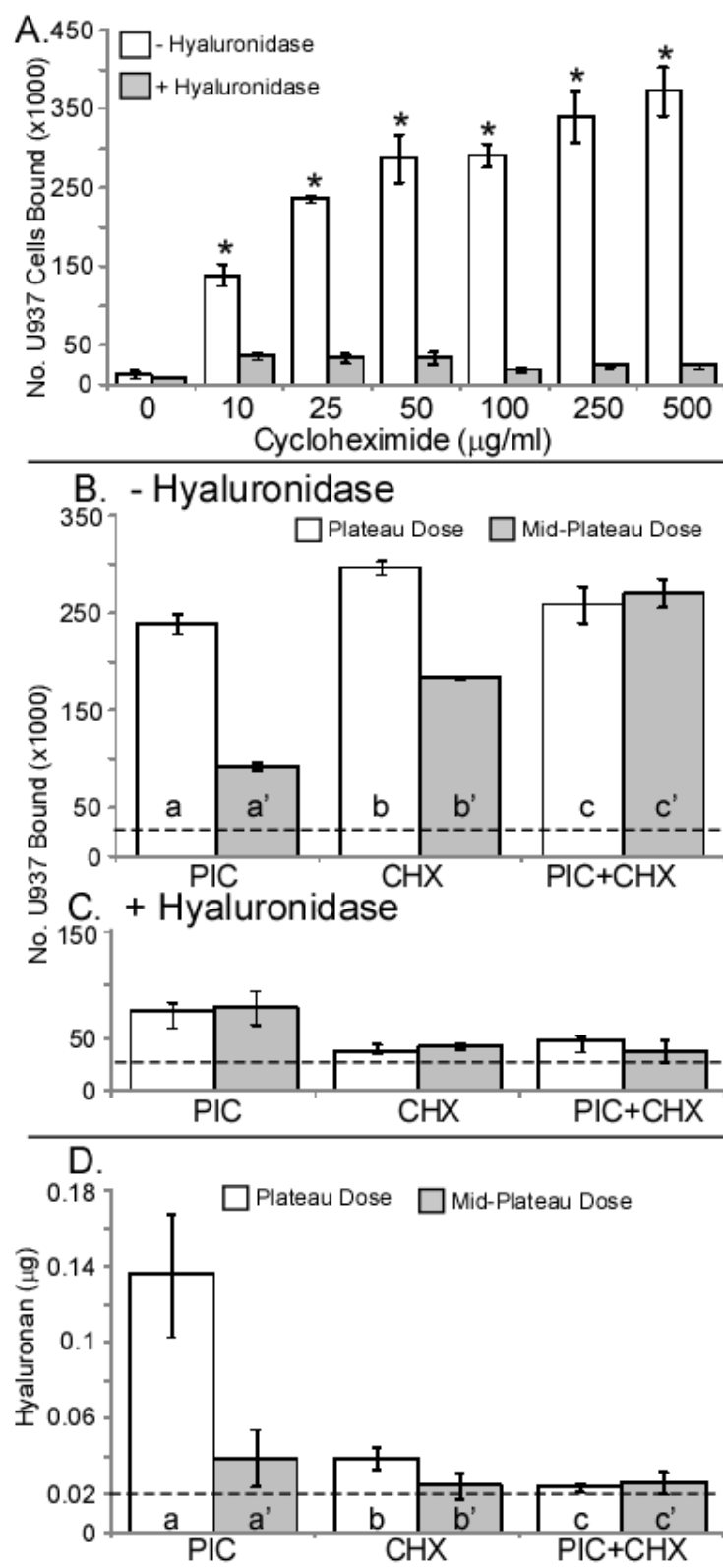


FIGURE 4

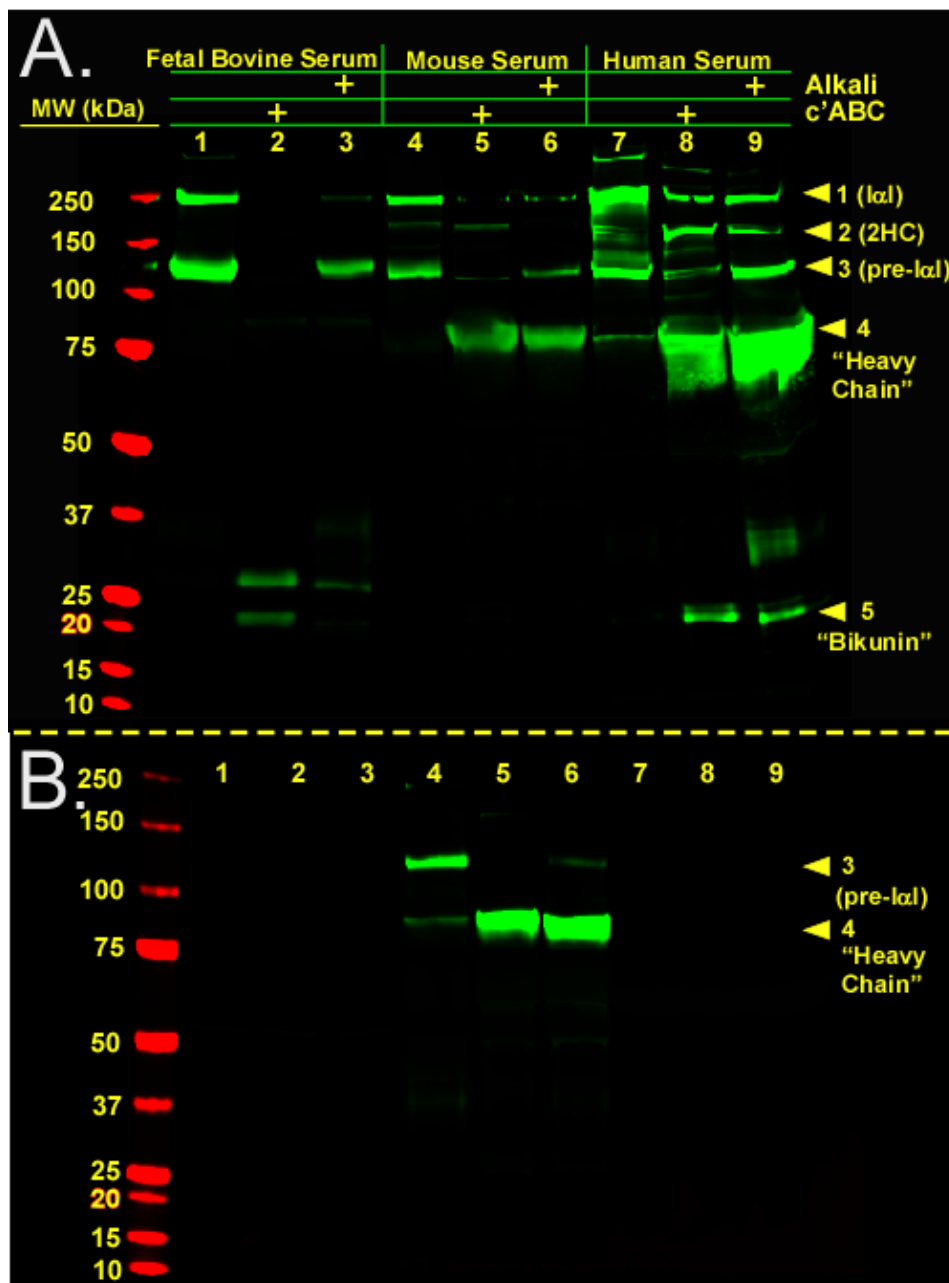


FIGURE 5

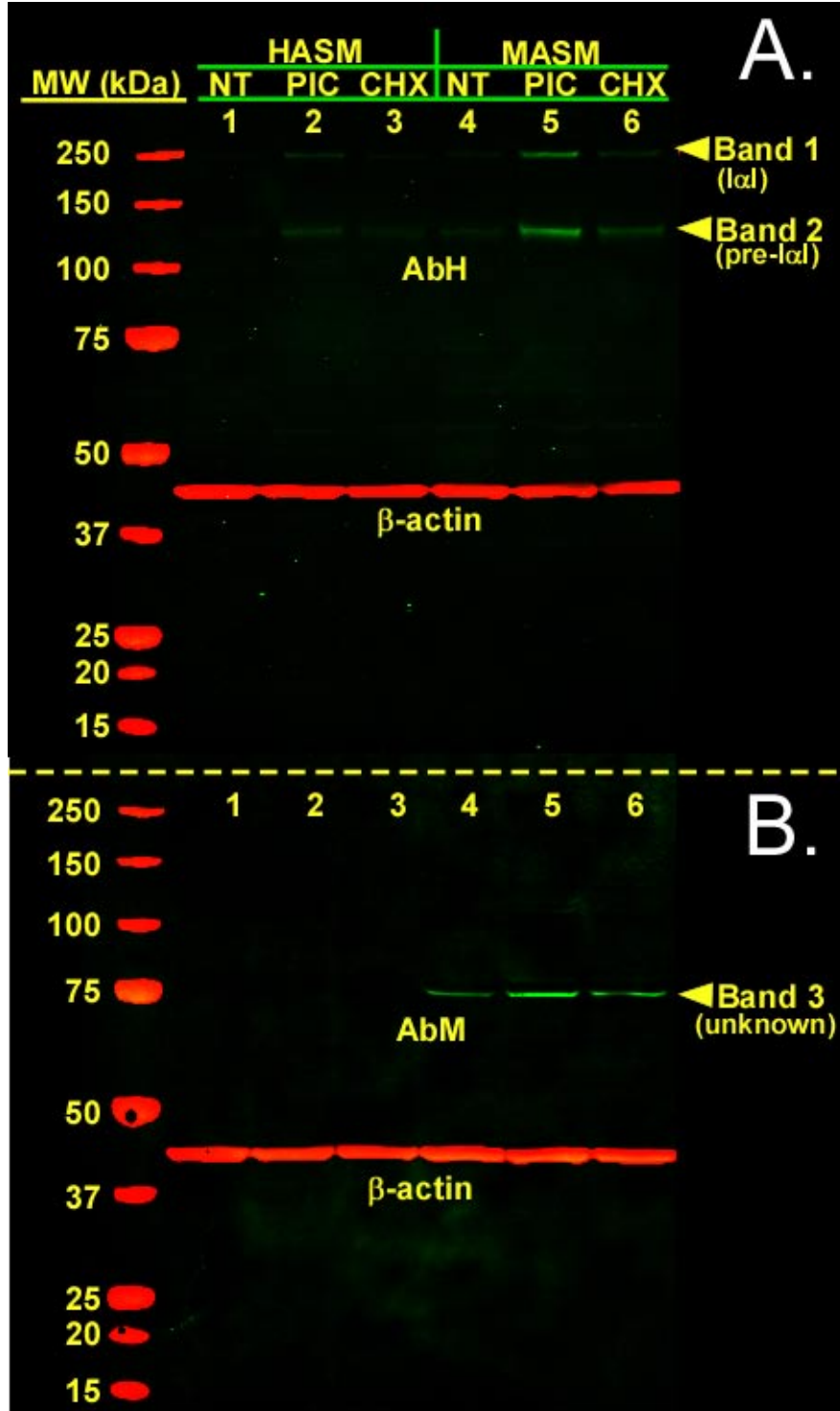


FIGURE 6

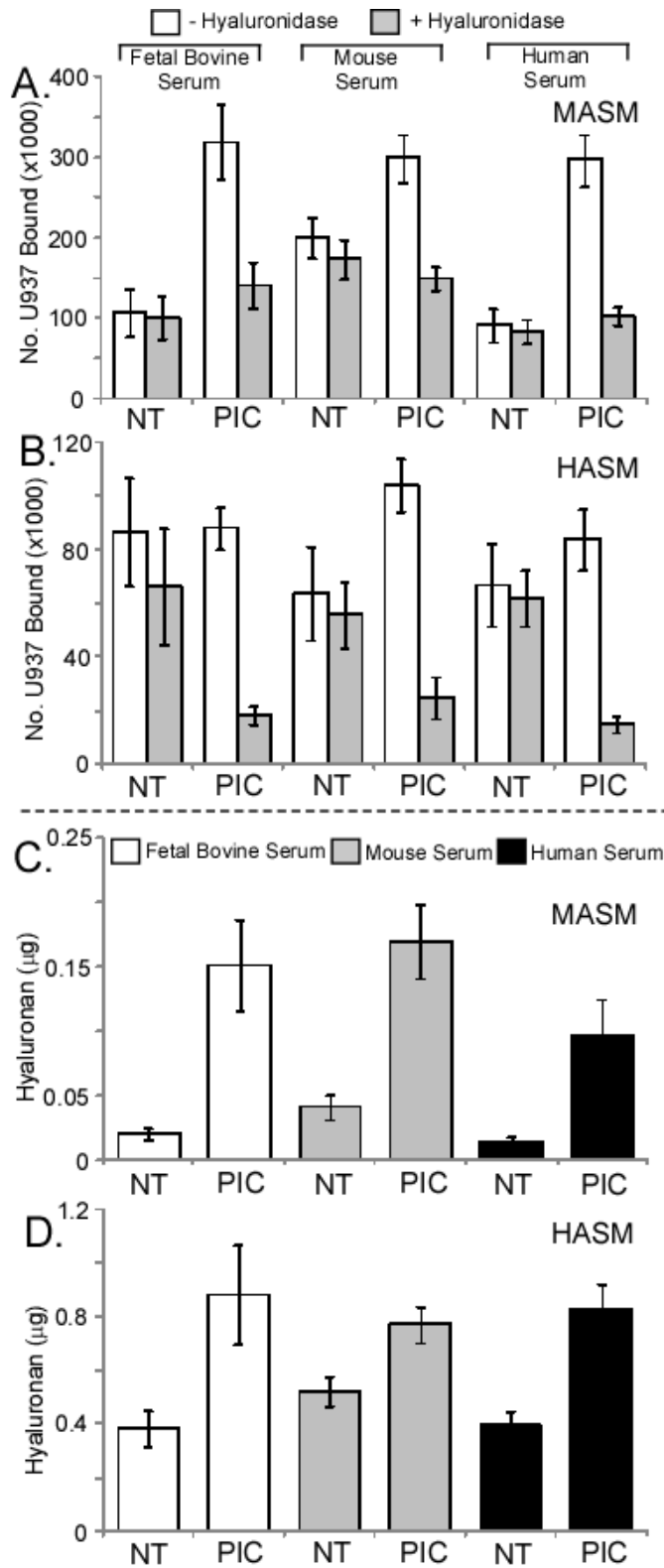


FIGURE 7

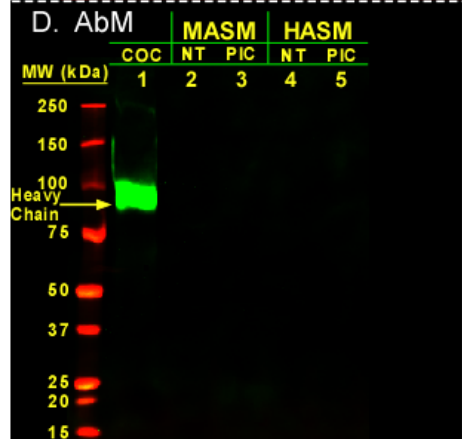
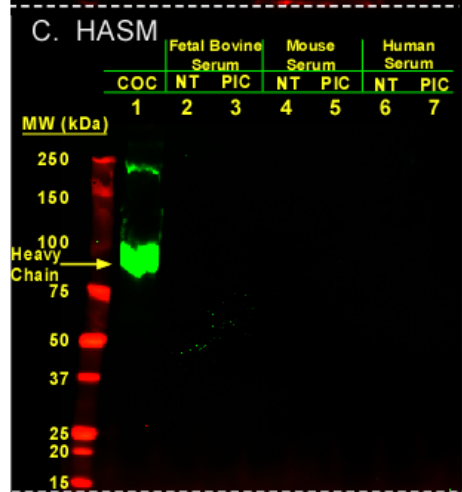
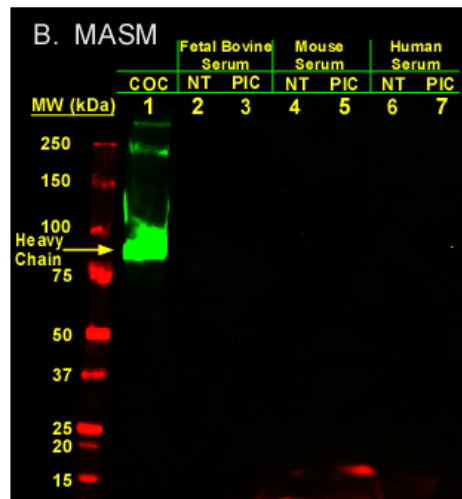
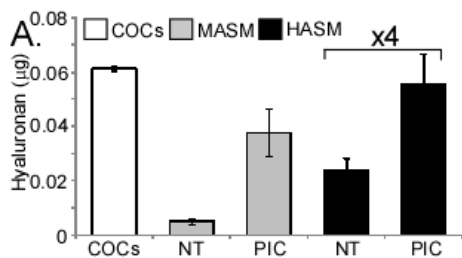


FIGURE 8

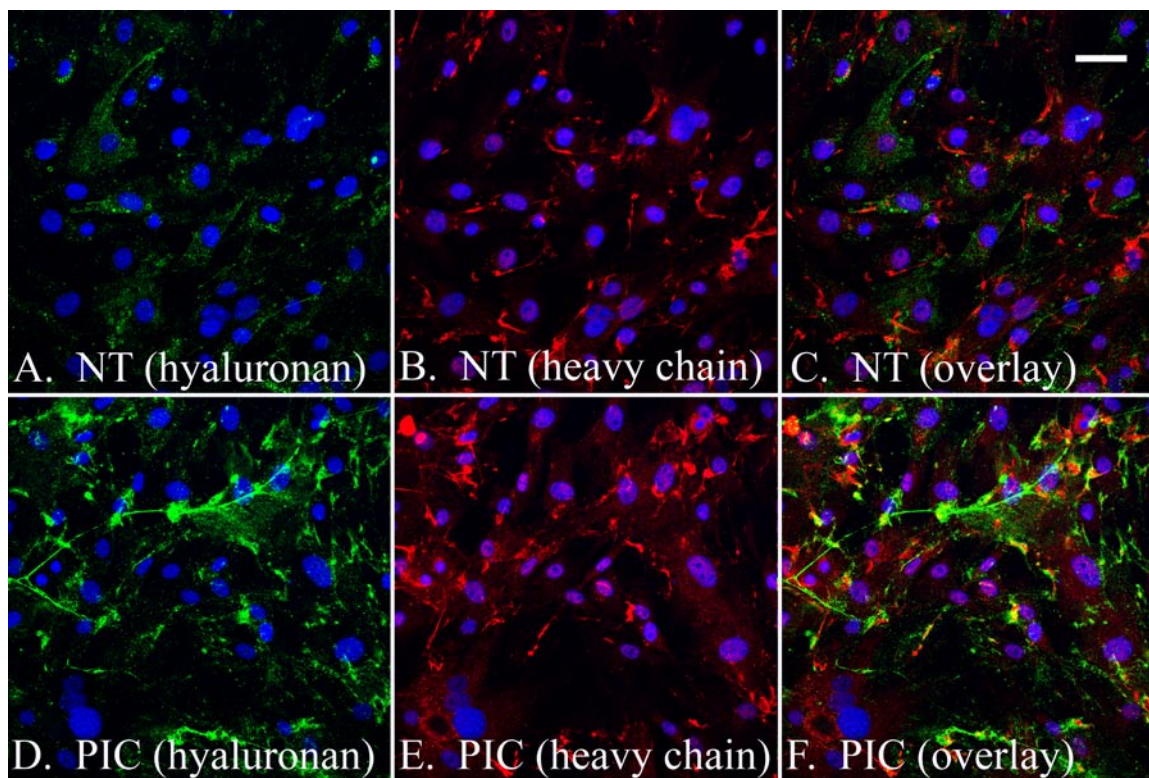


FIGURE 9

