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Decreased hyaluronan in airway smooth muscle cells from patients with asthma and COPD

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Short title
Decreased expression of HA in asthma and COPD

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Abstract

Glycosaminoglycans (GAG) are essential extracellular matrix molecules which regulate tissue flexibility, a parameter that is reduced in airways of patients with asthma and COPD. Here, we investigated the expression of GAG and their metabolizing enzymes in primary human airway smooth muscle cells obtained from healthy donors (controls) and patients with asthma or COPD.

Total GAG synthesis was assessed by [^{3}H]-glucosamine incorporation. GAG were isolated, purified, fractionated by electrophoresis and characterized using specific GAG-degrading enzymes. Secretion of hyaluronic acid by airway smooth muscle cells from patients with asthma or COPD was significantly decreased, as compared to controls. RT-PCR analysis and western blotting revealed that this decrease was associated with a significant reduction in the expression of hyaluronic acid synthase -1 and -2 and a significant increase of hyaluronidase 1. Furthermore, the expression of the hyaluronic acid receptor CD44 was significantly decreased, whereas the receptor for hyaluronic acid-mediated motility was not expressed in asthma or COPD.

Our results indicate that there is a decreased expression of hyaluronic acid in asthma and COPD associated with a synergistic regulation of hyaluronic acid metabolizing enzymes that may regulate the pathologic airway remodeling in these lung diseases.

Keywords

airway smooth muscle cells, asthma, COPD, glycosaminoglycans, CD44, hyaluronic acid

Introduction

Recent reports on asthma and chronic obstructive pulmonary disease (COPD) research provided clear evidence that the pathologies of both diseases can not be solely explained on the basis of a deregulated immune-response, and that malfunction of structure forming cells and disturbance of the homeostasis of extracellular matrix (ECM) molecules contribute significantly to the pathology of both diseases and reflect to airway remodeling (1, 2). Tissue remodeling describes the structural alterations that occur in the lung due to prolonged chronic inflammation within the airways, and involves qualitatitive or quantitative changes in cell density and the composition of the ECM in the pulmonary epithelium, the basement membrane, and the submucosa. Consequently, this modification in the ECM affects airway resistance, compliance, and elasticity, leading eventually to loss of lung function (1, 2).

Recent studies clearly indicated the outstanding contribution of airway smooth muscle cells (ASMC) to the pathology of asthma and COPD (1-5). Furthermore, clinical studies demonstrated that the reduction of ASMC in asthma patients by thermoplasty improved their quality of life and reduced symptoms and airway inflammation in long term (6, 7), thereby, supporting the eminent role of ASMC in this pathology. Hyperplasia of ASM bundles is a prominent pathology of the large-medium sized airways in asthma and of small airways in COPD (2). Furthermore, we have shown that ECM-associated glycosaminoglycans (GAG) play a central role in regulating the response of ASMC to mitogenic stimuli (8).

GAG are essential constituents of the ECM of the lung and possess important functional properties. In humans seven GAG have been identified: chondroitin sulfate A (CSA), dermatan sulfate (DS), chondroitin sulfate C (CSC), heparin, heparan sulfate (HS), hyaluronic acid (HA), and keratan sulfate (KS), and their function varies within the organ they are located. In the human lung KS was found on the apical surface of ciliated epithelial cells, CS and DS were secreted by epithelial and submucosa gland cells, and HS was reported in the ECM of tracheal tissue sections (9). HA and the enzymes which metabolize it are also endogenous to the pulmonary environment, and HA has been isolated from the lungs of mammals (sheep, guinea pig, rat) (10) and human lung parenchyma and pleura (11). In the lungs, the HA content is 15–150 mg/g dry weight (species specificity), which is mainly localized in the peri-bronchial and inter-alveolar/peri-alveolar tissue (12). The quantity of HA in human lung secretions was found to be approximately 66 ng/ml with values ranging from 34 to 423 ng/ml (13).

HA is a linear polysaccharide chain, composed of repeating disaccharide units of N-acetyl-D-glucosamine-β (1→4) - D-glucuronic acid-β (1→3), which exists in both a high molecular mass form (1–6 x 10^6 Da) and a polydisperse lower molecular mass form (0.1–0.5 x 10^6 Da), the latter predominating under inflammatory conditions (14). Polymerization of HA is regulated by the action of one or more of three HA synthases (termed HAS1, HAS2, and HAS3) (15), through the joining of the glycosidic residuals to the reducing chain extremity. HA is metabolized by

hyaluronidases (HYAL), mainly by HYAL1 and HYAL2, present in various tissues, including the lung (13). The effects of HA are exerted mainly through interactions with the HA receptor CD44, which is the main receptor mediating HA signaling (16), but also by RHAMM (receptor for HA-mediated motility) (17). HA receptors are expressed by lung fibroblasts (18) smooth muscle and endothelial cells of normal tissue (19). HA has diverse biological functions in migration and proliferation (8), embryonic development, tissue morphogenesis, cell growth, differentiation and ovulation (20), as well as in disease progression (21). However, reports on the functional role of HA in chronic inflammatory lung diseases are conflicting. This may be attributed to the fact that most studies on GAG expression in chronic inflammatory lung diseases are hindered by the lack of healthy lung tissue being used as the basic control condition.

In the present study, we have used primary airway smooth muscle cells (ASMC) from healthy lung tissue (control) and from patients with asthma or COPD. We investigated the expression of HA in these primary cells, and report that there is decreased expression of HA in ASMC from patients with asthma and COPD as compared to control. This decrease is associated with a reduced expression of HAS1 and HAS2 and an increased expression of HYAL1 in gene and protein levels. In addition, we found that RHAMM was expressed only by ASMC from controls.

Methods

Cell cultures

Primary cultures of ASMC were established from dissected airway muscle bundles obtained from isolated bronchi of 10 control subjects (organ donors), or from endo-bronchial biopsies of 11 patients with mild to moderate asthma, and 6 patients with COPD, as described earlier (22). Informed written consent was obtained from each patient as well as approval by the Human Ethics Committee of the University of Sydney and the Central Sydney Area Health Service. The available clinical characteristic of the patients including age, gender, diagnosis, FEV1 and medical treatment prior sampling are shown in Table 1. All patients included in this study have been diagnosed with asthma or COPD following the GINA and GOLD standard definition.

ASMC were counted and seeded at a density of 100,000 cells/cm^2 in 175 cm^2 flasks for GAG extraction and in 25cm^2 flasks for mRNA extraction and for cell counting. ASMC were characterized by positive immuno staining for α-smooth muscle cell actin, and calponin, as described earlier (1). ASMC were grown in DMEM medium supplemented with 5% FCS, 1% MEM-vitamins, 8 mM stabilized L-glutamine and 10 mM HEPES buffer (GIBCO BRL, Life Technologies, Sydney, Australia). For all experiments cells were used between passage 4-9; cells were grown until 80% confluence, and were serum-deprived prior to experiments for 24 h in medium containing 0.1% FCS. Unless otherwise stated, cells were routinely stimulated with 5% FCS and incubated for 24 h. Assays were performed on samples prior to stimulation (0 h of incubation) and on samples 12 h and 24 h after stimulation with 5% FCS. Cells in the presence of 0.1% FCS are assumed to be under non-inflammatory conditions, while stimulation with 5% FCS, is assumed to mimic an inflammatory condition. Comparisons described thereon are either between ASMC of different origin, or between 0.1% and 5% FCS for a particular type of ASMC.

[^{3}H]-Glucosamine incorporation

To measure de novo GAG synthesis, subconfluent ASMC were incubated with medium containing either 0.1 % or 5 % FCS in the presence of [^{3}H]-glucosamine (0.5 µCi/ml) (Amersham Biosciences, UK) for 24 h. Incorporation of [^{3}H]-glucosamine into GAG was measured as previously described (23). In brief, culture medium was collected and cells were washed twice with ice-cold PBS and lysed with 200 µl RIPA buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2). The cell layer (cells and deposited ECM) and cell culture medium were collected separately. Samples were digested with 0.1 KU of pronase (*Streptomyces griseus*; Calbiochem, Lucerne, Switzerland) and total GAG were precipitated by adding a mixture of ethanol (80% final concentration) containing 1.3% (w/v) sodium acetate, (overnight, 4°C) and then centrifuged (10,000 x *g*, 15 min). The pellets were

dissolved in 0.5 M NaOH and total GAG synthesis was calculated on the basis of [^{3}H]-glucosamine incorporated into GAG.

Isolation, purification, fractionation and characterization of GAG

Cell culture media (20 ml) were collected separately from the cell layers, which were washed twice with 10 ml of ice-cold PBS and harvested by scraping. GAG were isolated and purified from the culture media and the cell layers, as previously described (24). In brief, lipids were extracted with 4 volumes of chloroform/methanol (1:2). Organic solvents were removed by centrifugation (3,200 x *g*, 20 min, 4°C), and the pellet was washed with 10 ml of ethanol, centrifuged (3,200 x *g*, 20 min, 4°C), and dried (4 h, 40°C). The pellet was resuspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), containing 1 mM $CaCl_2$ and the protein was digested with 0.1 KU of pronase (*S. griseus*; Calbiochem) (72 h, 60°C) by adding equal amounts of pronase at 24 h intervals. The pronase solution was preheated (30 min, 60°C) to eliminate any glycosidase activity. DNA digestion was accomplished by incubating with 400 KU of DNase I (EC 3.1.21.1; Calbiochem) (16 h, 37°C). After adjustment of the $CaCl_2$ concentration to 1 mM the reaction was stopped by 0.1 KU of pronase (60°C, 24 h). The samples were then titrated with 10 mM NaOH to pH 10.0-11.0, and incubated (16 h, 45°C) in the presence of 1 M $NaBH_4$. Samples were neutralized with 50% (v/v) acetic acid and the extracted GAG were precipitated by the addition of 4 volumes of ethanol in the presence of 0.1 volume of 3 M CH_3COONa (overnight, 4°C). GAG were recovered by centrifugation (2,000 *x g*, 20 min), and the pellets were dissolved in double distilled H_2O and stored at 4°C. Colorimetric determination of uronic acids was performed according to Bitter and Muir (25).

Fractionation of total GAG

Fractionation of GAG was achieved by electrophoresis on cellulose acetate membranes as described earlier (24). In brief, 2 µl of the GAG solution, containing 4 µg of uronic acids, were placed at the origin (10 mm from the cathode side) of a cellulose acetate strip. Electrophoresis was carried out in 100 mM pyridine / 470 mM formic acid (pH 3.0) at 7 mA constant current (70 min, room temperature). After electrophoresis, cellulose acetate membranes were stained with 0.2% Alcian blue (w/v), in 0.1% acetic acid (v/v), for 10 min and washed with 0.1% acetic acid (v/v) for 20 min. The intensity of the staining was quantified by the computer-assisted image analysis program (Eastman Kodak, Rochester, NY).

Treatment of the purified glycans with GAG-degrading enzymes

Speed-vacuum-dried GAG (5 µg uronic acids) were incubated in a final volume of 15 µl of either: (a) Heparinase: samples dissolved in 100 mM Tris-HCl buffer (pH 7.0) containing 3 mM $CaCl_2$ and incubated (15 h, 30^{o}C) with $4x10^{-4}$ U heparin lyase I (EC 4.2.2.7, *Flavobacterium*

heparinum, Seikagaku, Tokyo). (b) Heparitinase: samples dissolved as above were incubated (16 h, 43^{o}C) with 4x10^{-4} U heparan sulphate lyase (heparitinase: EC 4.2.2.8, *Flavobacterium heparinum*, Seikagaku, Tokyo). (c) Chondroitinase ABC: samples dissolved in 100 mM Tris-HCl buffer (pH 8.0) containing 50 mM sodium acetate were incubated(16 h, 37^{o}C) with 2 x 10^{-4} U chondroitin ABC lyase (EC 4.2.2.4, *Proteus vulgaris*, Sigma-Aldrich Chemie, Steinheim, Germany). (d) Chondroitinase B: samples dissolved in 100 mM Tris-HCl buffer (pH 7.4) were incubated (16 h, 37^{o}C) with 0.1 U chondroitin B lyase (*Flavobacterium heparinum*, Sigma). (e) Keratanase: samples dissolved in 50 mM Tris-HCl buffer (pH 7.4) were incubated (16 h, 37^{o}C) with 0.05 U keratan sulphate endo-β-D-galactosidase (EC 3.2.10.3, *Pseudomonas species*, Sigma). (f) Hyaluronidase: samples dissolved in 20 mM sodium acetate, buffered with acetic acid to pH 5.0, were incubated (14 h, 60^{o}C) with 4 U hyaluronate lyase (EC 4.2.2.1, *Streptomyces hyalurolyticus*, Sigma). Incubation times and enzyme concentrations were as required for complete degradation of standard substrates, as previously published (26). Substrates incubated separately with their respective buffers served as controls. Digestion was evaluated by electrophoresis on cellulose acetate membranes and quantified by the computer-assisted image analysis programme of Kodak.

Measurements of HA

A) Net amount of hyaluronic acid secreted by primary ASMC: Cells were grown in 24-well plates, washed twice with culture medium to remove HA accumulated during cell growth, and incubated for 24 h. At the end of incubation time, aliquots of cell culture medium were collected and tested for the quantity of HA by ELISA (Corgenix, Westminster, CO). Briefly, ELISA plates coated with HA binding protein were incubated with samples or standards (1 h, room temperature) in duplicates, washed five times with washing buffer, incubated with a solution containing horseradish peroxidase-conjugated HA-binding protein (30 min, room temperature), washed again five times, and incubated with 100 µl of the substrate solution. After 30 min, the reaction was stopped by adding an equal amount of sulfuric acid (0.36 N), and the OD was measured at 450 nm (630-nm reference).

B) Relative amount of HA in total GAG: Total GAG were isolated and purified from the cell culture medium and the cell layers as described above and the relative amount of HA was measured in aliquots containing 0,1 µg of uronic acids by ELISA (Corgenix, UK), as described above.

Polyacrylamide gel electrophoresis (PAGE)

Total GAG (4 µg uronic acids) isolated and purified from the culture medium or the cell layers of ASMC were analyzed on 4% polyacrylamide gels, as previously described (26). HA of 225 kDa and chondroitin sulfates of 29 and 57 kDa were used as molecular weight markers. The

molecular mass of the markers was previously determined by analytical ultracentrifugation (27). Gels were stained with a solution of 0.5% (w/v) Alcian blue, dissolved in 25% (v/v) isopropyl alcohol and 1% (v/v) acetic acid, for 12 h. The same solution without the dye was used for destaining.

RT-PCR

RNA was extracted from cells using the RNeasy (Qiagen, Hilden, Germany). Total RNA was subjected to reverse transcription using MMLV-RT (Invitrogen GmbH, Life Technologies, Karlsruhe, Germany). Five µl of the reaction mixture were subjected to PCR amplification in 50 µl reaction volume, containing 25 pmoles of relevant primers, 200 µM dNTPs (Invitrogen GmbH, Life Technologies, Karlsruhe, Germany), 2 mM $MgCl_2$ and 1 unit of Taq DNA polymerase in 1x Taq DNA polymerase buffer (Promega, Madison, WI), on a PTC-100 Thermal Controller (MJ Research Inc., Watertown, MA). All primer sequences and the PCR conditions are listed in Table 2. PCR products were analyzed on a 2% (w/v) agarose gel. DNA bands were visualized in ethidium bromide-stained gels under UV-light and quantified on the basis of β-actin mRNA expression, which was amplified under non saturating conditions using the computer-assisted image analysis program of Kodak (Eastman Kodak, Rochester, NY).

Western blot analysis

Total protein extracts were prepared from 80% confluent ASMC. Ten µg of proteins were dissolved in Laemmli buffer, denatured (95^{o}C, 5 min), chilled on ice (5 min), centrifuged (13,000 x g, 50 sec), and applied to electrophoresis on 4–15% SDS-PAGE. Proteins were transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA) by over night transfer at 50^{o}C, which was confirmed by staining with Coomasie Blue. The membranes were then washed three times with PBS, blocked with 5% skimmed milk in PBS (4^{o}C, overnight), and incubated with one of the primary antibodies (all from Santa Cruz: CD44: sc-59909, RHAMM: sc-16170, HYAL1:sc-101340, HAS1: sc-23145, HAS2: sc-66916) overnight at 4^{o}C. The membranes were then washed three time (5 min each) with blocking buffer and incubated with a secondary antibody at room temperature for 90 min (all from Santa Cruz: for CD44: sc-2005, for RHAMM: sc-2020, for Hyal-1: sc-2005, for HAS1: sc-2020, for HAS2: sc-2004). Before bands were visualised the membranes were washed three times with PBS and then soaked in SuperSignal West Pico Chemilluminescent Substrate (cat 34077, Pierce). To visualize the protein bands the membranes were exposed to Bio-Max-ray films (Kodak).

Protein determination

The protein content was determined in aliquots of cell culture medium by standard Bradford assay (Bio-Rad, Glattbrugg, Switzerland) using bovine serum albumin (Sigma) as standard.

Statistical analysis

The computer software SPSS 16.0 (SPSS Inc.) was used for all statistical calculations and analyses. Normal distribution of data was checked using Kolmogorov-Smirnov analysis. All parametric data were analyzed with analysis of variance (ANOVA) for repeated measurements. If significant, ANOVA was followed by post-hoc multiple comparisons between the CTRL and other groups by Dunett's test. Non parametric data were analyzed with the Kruskal Wallis test while Friedman's test was used for related samples. Two-tailed levels of significance were used in all statistical calculations. Reproducibility of measurements was checked with the coefficient of variation factor. All data are expressed as mean values ± standard error (SEM) of the mean. Difference was considered to be statistically significant at (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$.

Results

Characterization of ASMC

Under light microscopy, ASMC from controls, asthmatics and COPD patients appeared spindle-shaped, with central oval nuclei containing prominent nucleoli, and displayed the typical "hill and valley" proliferation pattern in culture (data not shown). All cells showed uniform staining for both the smooth muscle–specific contractile proteins α-smooth muscle actin and calponin, as previously described (1), indicating that these cells were ASMC.

Total GAG secretion and deposition by ASMC

Measurements of total GAG synthesis by [^{3}H]-glucosamine incorporation revealed that under non-inflammatory conditions (cells in the presence of 0.1% FCS) there were no significant differences in the secretion and deposition of total GAG by ASMC between controls and patients with asthma or COPD (Fig. 1A). However, under inflammatory conditions (cells stimulated with 5% FCS) secretion of total GAG was increased in all three groups (Fig 1A) but this effect was significant only for secreted GAG by control ASMC (2150 ± 250 cpm for 0.1% FCS, versus 3151 ± 625 cpm, for 5% FCS $p < 0.05$; Fig. 1A).

Furthermore, when stimulated with 5% FCS, ASMC from patients with asthma or COPD secreted significantly less GAG than ASMC from controls (3240 ± 475 for controls, 2310 ± 315 for asthma and 1980 ± 325 for COPD, $p < 0.01$ for control versus asthma and $p < 0.05$ for control versus COPD, Fig. 1A).

Identification of GAG in ASMC

Electrophoresis on cellulose acetate membranes of 4 µg of uronic acids of total GAG isolated from the cell culture medium of control ASMC 24 h after stimulation with 5% FCS resulted in four distinct GAG populations, assigned as G1, G2, G3 and G4 (Fig. 1B), which migrated with the same mobility as HA, HS, DS and CS, respectively. Enzymatic treatment with specific GAG-degrading enzymes (Table 3) confirmed that G1 is HA, G2 is HS, G3 is DS and G4 is CSA and/or CSC. The same GAG were also identified in the cell culture medium of ASMC obtained from asthma and COPD patients (Fig. 1B) indicating that there are no qualitative differences in the nature of total GAG secreted by ASMC from controls and patients with asthma or COPD. However, quantitation of the Alcian Blue staining with a computer-assisted image analysis program revealed that HA secretion was significantly decreased in the cell culture medium of ASMC from patients with asthma and COPD, as compared to controls (Fig. 1C).

We further analyzed the GAG deposited in the cell layers of ASMC from healthy lung tissue and patients with asthma or COPD. Three distinct GAG populations were identified, which were characterized by enzymatic treatment as HA, HS and DS (Fig. 1B, Table 3). Quantitation of the intensity of the Alcian blue staining indicated that the amount of HA which was deposited in

the cell layers of ASMC was significantly decreased in asthma and COPD, as compared to controls (Fig. 1C).

Reduced secretion of HA by ASMC from patients with asthma and COPD

Since HA was the most abundant GAG secreted or deposited by primary ASMC from all three groups, and since there were indications from the Alcian blue staining of cellulose acetate membranes that HA was decreased in asthma and COPD we further measured the net amount of HA secreted by ASMC after 12 and 24 h of incubation by ELISA. Compared to control ASMC from asthma or COPD patients secreted significantly lower amounts of HA after 12 h (3.7 ± 0.25 μg HA/1000 cells for controls, 1.6 ± 0.17 μg HA/1000 cells for asthma and 1.5 ± 0.25 μg HA/1000 cells for COPD, $p < 0,01$, Fig. 2A) and 24 h of incubation (5.1 ± 0.35 μg HA/1000 cells for controls, 3.8 ± 0.30 μg HA/1000 cells for asthma and 2.7 ± 0.25 μg HA/1000 cells for COPD, $p < 0.02$ και $p < 0,01$ for asthma and COPD, respectively, Fig. 2A).

The relative content of HA in 0.1 μg of uronic acids of total secreted or deposited GAG was also measured by ELISA. We observed that the amount of HA which was measured in secreted GAG was significantly lower in asthma (6.32 ± 0.8 ng/0.1 μg of uronic acids, $p < 0.01$) and COPD (7.57 ± 1.8 ng/0.1 μg of uronic acids, $p < 0.02$) as compared to control (11.77 ± 1.5 ng/0.1 μg of uronic acids) (Fig. 2B). Furthermore, the amount of HA which was measured in GAG deposited in the cell layers was significantly lower in asthma (4.55 ± 0.6 ng/0.1 μg of uronic acids, $p < 0.01$) and COPD (6.12 ± 1.4 ng/0.1 μg of uronic acids, $p < 0.02$), as compared to control (9.72 ± 1.2 ng/0.1 μg of uronic acids) (Fig. 2B).

Gene and protein expression of HAS1 and HAS2 decreases whereas expression of HYAL1 increases in asthma and COPD

Since HA secretion and deposition was decreased in asthma and COPD we sought to further investigate the expression of HA metabolizing enzymes by RT-PCR. As shown in Fig. 3A, ASMC of different origin express *Has1*, *Has2* and *Has3*. Quantitation of the PCR results by an image analysis program revealed that the expression of *Has1* was significantly decreased in ASMC from asthma patients at 0, 12 or 24 h ($p < 0.05$) or from COPD patients after 24 h of incubation ($p < 0.05$), as compared to control (Fig. 3B). *Has2* mRNA expression was also significantly decreased in ASMC from asthma or COPD patients ($p < 0.05$), as compared to control (Fig. 3C). There were no significant differences for *Has3* expression between ASMC of different origin (Fig 3D). Immunoblot expreriments using antibodies against HAS1 and HAS2 showed that protein expression of both enzymes was reduced in asthma and COPD as compared to controls (Fig. 3E), confirming the results obtained from the RT-PCR analysis.

Hyal1, *Hyal2* and *Hyal3* were also expressed in ASMC from all three groups (Fig. 4A). Quantitation of the PCR results revealed that the expression of *Hyal1* (Fig. 4B) was increased in

ASMC from asthma at 24 h ($p < 0.05$) and from COPD patients at 0, 12 and 24 h ($p < 0.01$), as compared to control. There were no significant differences in the expression of *Hyal2* (Fig. 4C) or *Hyal3* (Fig 4D) between ASMC of different origin. Immunoblot expreriments using antibodies against HYAL1 showed that protein expression of this enzyme was induced in asthma and COPD as compared to controls (Fig. 4E), confirming the results obtained from the RT-PCR analysis.

HA in asthma and COPD has a lower molecular mass than controls

We further investigated if the differential expression of HYAL1, HAS1 and HAS2 in ASMC from asthma or COPD patients resulted in HA of different molecular mass, as compared to controls. We performed PAGE analysis of 4 µg of the total GAG isolated and purified from ASMC of different origin and compared to GAG of known molecular mass (Fig. 5). The migration of HA was identified after treatment of the samples with hyaluronidase prior to PAGE (data not shown). We found that HA isolated from the cell layers of control ASMC after stimulation with 5 % FCS for 24 h migrated with an average molecular mass > 700 kDa, whereas HA of asthma and COPD ASMC exhibited a lower average molecular mass of 250 kDa (Fig. 5). Similar results were obtained for HA isolated from the cell culture medium. These results indicate that asthma and COPD are associated with the presence of HA in the lung which has a lower molecular mass than HA in healthy lungs.

Disease-specific gene and protein expression of HA receptors CD44 and RHAMM by ASMC

Furthermore, we investigated the transcription of HA receptors by ASMC. RT-PCR analysis revealed that *CD44* was constitutively expressed by ASMC of different origin (Fig. 6A). Quantitation of the PCR results revealed that the expression of *CD44* was reduced in cells of asthma patients and this result was statistically significant after 24 h of treatment with 5% FCS ($p < 0.05$, Fig. 6B). In ASMC from COPD patients, *CD44* mRNA level was significantly reduced after 12 h ($p < 0.02$) and 24 h of treatment with 5% FCS ($p < 0.01$, Fig. 6B). Immunoblot expreriments confirmed the results obtained from the RT-PCR analysis. As shown in Fig. 6D, CD44 protein expression was reduced in asthma and COPD as compared to controls.

RT-PCR analysis for the mRNA encoding for *RHAMM* revealed that it was expressed only by control ASMC (Fig. 6A). The expression of *RHAMM* in control ASMC significantly increased within 24 h of treatment with 5% FCS by almost 5-fold ($p < 0.01$) (Fig. 6C). Interestingly, ASMC of asthma or COPD patients did not express *RHAMM* at any time point investigated (Fig. 6A). These results were also confirmed by immunoblot expreriments. As shown in Fig. 6D, RHAMM protein was not expressed in ASMC obtained from patients with asthma and COPD.

Discussion

The pathogenesis of asthma and COPD includes chronic inflammation of the airways and airway remodeling. Major features of the remodeling processes include fibrosis in the sub-epithelial regions and the nearby interstitial tissue of the airways, myocyte hypertrophy and hyperplasia, myofibroblast hyperplasia, mucous metaplasia, vascular abnormalities and thickening of the airway wall (2). However, several issues of airway remodeling associated with asthma and COPD remain to be clarified. These include the sequence of the molecular and cellular events involved, the contribution of each facet of the remodeling processes to the clinical symptoms and pathology, and the possibility that airway remodeling represents a healing and repair response to aspects of the pathogenesis. Furthermore, the precise contribution of the individual ECM molecules to airway remodeling that generates the asthma or COPD phenotype has not been adequately defined. Here, we attempted to clarify the latter, and we present data on the differential turnover of HA in AMSC of different origin, employing healthy lung tissue as the basic control condition. ASMC obtained from patients with asthma or COPD secreted lower amounts of fragmented HA, and this was associated with decreased gene and protein expression of HAS1 and HAS2, increased gene and protein expression of HAYL1, decreased gene and protein expression of CD44 and lack of the HA receptor RHAMM, as compared to ASMC from normal lung tissue. Lower levels of HA in ASMC implicate a reduction of tissue water content and flexibility which may contribute to the extended broncho-constriction and stiffness of the airways in asthma and COPD.

Heparan, dermatan and chondroitin sulfates, as well as HA are present in ASMC from controls and patients with asthma or COPD. These results are in agreement with the reported presence of heparan, chondroitin and dermatan sulfates in tracheal tissue sections (9) and of HA in the human lung (11-13). However, we observed that total GAG synthesis was reduced in ASMC from asthma and COPD patients when compared to controls. This may be ascribed to the decreased net content of HA or to the decreased concentration of HA relative to total GAG in diseased ASMC. The decreased secretion or deposition of HA was apparently due to both a decrease in HA synthesis and an increase in HA degradation, since RT-PCR analysis and immunoblotting revealed a significant reduced expression of HAS1 and HAS2 and a significant increased expression of HYAL1 in ASMC from patients with asthma and COPD, as compared to controls. Our results provided evidence that reduced levels of HA are associated with asthma and COPD.

How could reduced levels of HA contribute to the pathogenesis of asthma and COPD? Indeed, there is considerable evidence that HA has a pleiotropic protective role in the lung. HA possess a unique capacity to link and retain water molecules in the inter-fibrillar space, via osmotic pressure and flow resistance, and thus contributes to the structure of the amorphous colloidal matrix which glues together cells and connective fibres (10). This provides HA with the

ability to hydrate and control solute transport and microcirculatory exchanges, due to its influence on interstitial volume, hydraulic conductibility and macromolecule diffusion (28). Other physiological functions of HA include the interaction with proteins by sieve and exclusion effects (barrier effect), stabilization of the ECM structure by electrostatic interactions, lubrication through its rheological properties, increased mucociliary clearance and prevention of elastin degradation (10). Furthermore, in the healthy lung HA stimulates ciliary clearance, retains homeostatic enzymes at the apical surface, and binds and stabilizes lung surfactant molecules (29). HA stabilizes proteoglycans in the ECM (30), contributes to tissue repair (20), inhibits migration, chemotaxis and aggregation of polymorphonuclear leucocytes and monocytes (31) and prevents elastase degradation of pulmonary elastin by a mechanism of protective coating (32).

The above biological functions of HA point to a protective role in the bronchial tissue, which correlates with our observation that HA is decreased in ASMC from asthma and COPD patients. In this context, HA blocked acute bronchoconstriction caused by human neutrophil elastase in sheep (33), and a single dose of inhaled HA was suggested to protect against exercise-induced bronchoconstriction in asthma patients (12). Furthermore, in COPD patients (34) or elastase-induced emphysema (35) treatment with HA had beneficial effects. It is of interest that two other GAG, heparin and heparan sulfate have also been reported to be beneficial during asthma therapy by a mechanism of action that it is not directly related to their anticoagulant property (36).

In contrast to the protective role of HA in lung physiology, it has also been reported that serum levels of HA did not differ between asthma or wheeze patients compared to normal controls (37), that inhaled low molecular mass HA (0.15×10^6 Da) did not significantly protect against exercise-induced broncho-constriction in asthmatic patients (38) and that there are increased levels of HA in lung secretions of asthma (39) and COPD (13) patients**.** However, these apparently contradictory reports may be explained as follows: (a) HA serum levels may not necessarily reflect HA levels in the lung; (b) it is the high molecular mass HA that exerts beneficiary effects; (c) lower molecular mass HA ($0.3–0.5 \times 10^6$ Da) predominate under inflammatory conditions (14); (d) the increased levels of HA in lung secretions of asthma and COPD patients may reflect enhanced degradation as a consequence of the increased expression of *Hyal2* in homogenized lung tissue of COPD patients (13) and *Hyal1* in ASMC that we report here and subsequent secretion of HA.

The argument for a protective role of HA of high molecular mass in the lung is further supported by reports that HA of high but not of low molecular mass inhibited the function of alveolar (40) and peritoneal macrophages (41) and that goblet cell metaplasia induced by reactive oxygen species in normal human bronchial epithelial cells was associated with HA depolymerization (42). Furthermore, HA of low but not high molecular mass prolonged the survival of eosinophils, stimulated the synthesis of transforming growth factor-β_1 *in vitro* (43), and

induced the expression of cytokines, chemokines and inducible NO synthase by macrophages (44).

With respect to the size of HA, we observed that ASMC from patients with asthma or COPD expressed HA of lower molecular mass, as compared to controls. This may be the result of: (a) the increased expression at gene and protein levels of HYAL1 in asthma and COPD and (b) the reduced expression of HAS1 and HAS2 since it has been shown that the catalytic rates and the final molecular weight product of HA are different for the three HAS isoforms (45). HAS1 is the least active, and produces HA of $0.2–2.0\times10^6$ Da, whilst HAS2 produces similar sized HA fragments but is more catalytically active. Finally, HAS3 produces smaller HA fragments no larger than 0.1×10^6 Da, and may be involved in activation of signal transduction (46).

The wide range of functions of HA in different cell types is mediated through its receptors, CD44 and RHAMM (16, 17). Our data show that CD44 was reduced in ASMC from asthma or COPD patients compared to controls, while RHAMM was not expressed in gene or protein level. It has been shown that CD44 is the major cell-surface hyaluronan receptor and is required to clear hyaluronan degradation products produced during lung injury (47). Therefore, it may be postulated that the reduced expression of CD44 that we report in asthma and COPD may be associated with impaired clearance of hyaluronan of low molecular mass from the lung, resulting in persistent inflammation.

HA also binds to RHAMM, which controls the effect of HA on cell migration, proliferation, and motility, apparently via RHAMM interaction with the cytoskeleton (48, 49). It remains to be elucidated if lack of RHAMM in asthma and COPD is associated with impaired function of HA in the lung which may result in pathophysiological changes leading to the diseases.

In conclusion, the available literature and the results presented here, using healthy lung tissue as the basic control condition, indicate that HA of high molecular mass is involved in physiological aspects of lung function, while it is the fragmented HA, due to reduced expression of HAS1, HAS2 and increased expression of HYAL1, which contributes to the inflammatory processes in asthma and COPD pathology.

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Figure legends

Figure 1. Total GAG secretion and deposition by primary ASMC from control, asthmatic and COPD patients. Subconfluent (80%) primary ASMC were incubated with 0.1 % or 5% FCS in the presence of [^{3}H]-glucosamine (0.5 µCi/ml) for 24 h. (A) Total GAG secretion and deposition in the ECM was determined as cpm. Experiments were performed in triplicate for each patient. Bars represent means ± SEM of 10 healthy donors, 11 patients with asthma, and 6 patients with COPD. *** = $p < 0.001$, [ ] = as compared to 0.1 % FCS, ( ) = as compared to respective control. (B) Representative analysis of the electrophoretic mobility on cellulose acetate membranes of GAG (G1 to G4) isolated and purified from primary ASMC of two healthy donors (Ct), two asthma patients and two COPD patients. Total GAG were isolated and purified 24 h after stimulation with 5% FCS from cell culture medium or cell layers. Migration of commercially available markers is indicated by arrows on the left: HA, hyaluronic acid; HS, heparan sulfate; DS, dermatan sulfate; CS, chondroitin sulfate. (C) Quantitation of Alcian blue intensity using a computer assisted analysis program. Each bar represents the mean ± SEM of triplicate determinations for each patient. (**) = $p < 0.01$, (***) = $p < 0.001$. Statistical differences indicated are between controls and asthma or COPD in cell culture medium and cell layers, respectively.

Figure 1

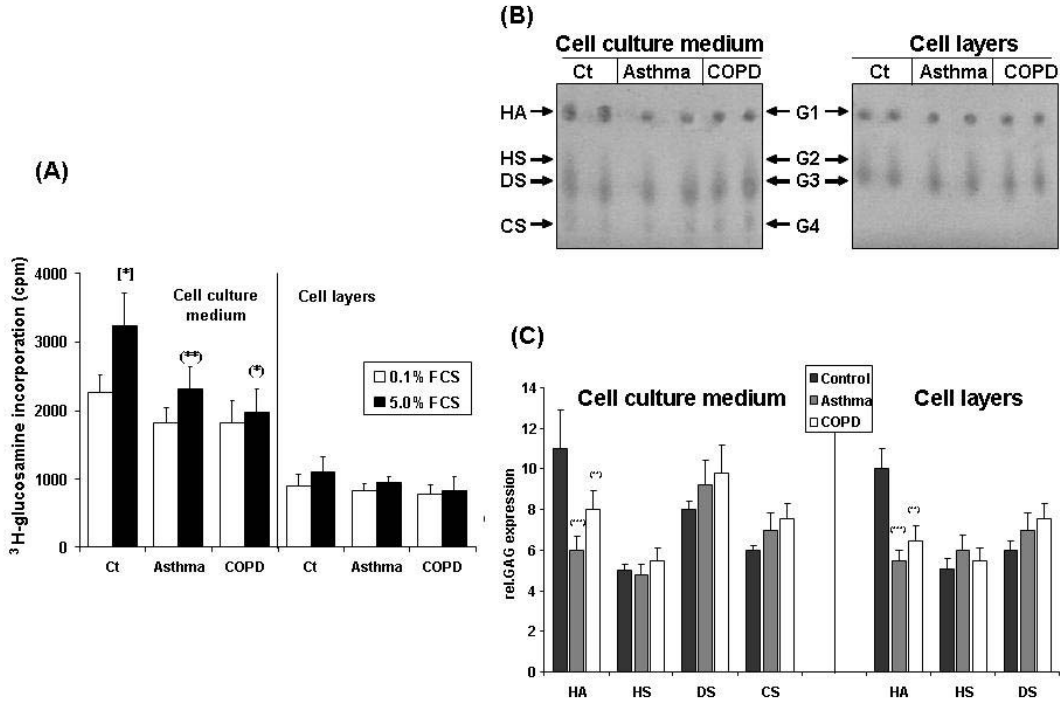

Figure 2. Disease-specific secretion and relative amount of HA by human ASMC. (A) The amount of secreted HA was measured by ELISA in aliquots of cell culture medium. (B) The relative amount of HA was determined by ELISA in aliquots of total GAG containing 0.1 µg of uronic acids. Determinations were performed in triplicate for each patient. Bars represent means ± SEM of 10 healthy donors (Ct), 11 patients with asthma, and 6 patients with COPD. (**) = $p < 0.01$, (***) = $p < 0.001$. Statistical differences indicated are between controls and asthma or COPD at each time point for (A) and between controls and asthma or COPD in cell culture medium and cell layers, respectively for (B).

Figure 2

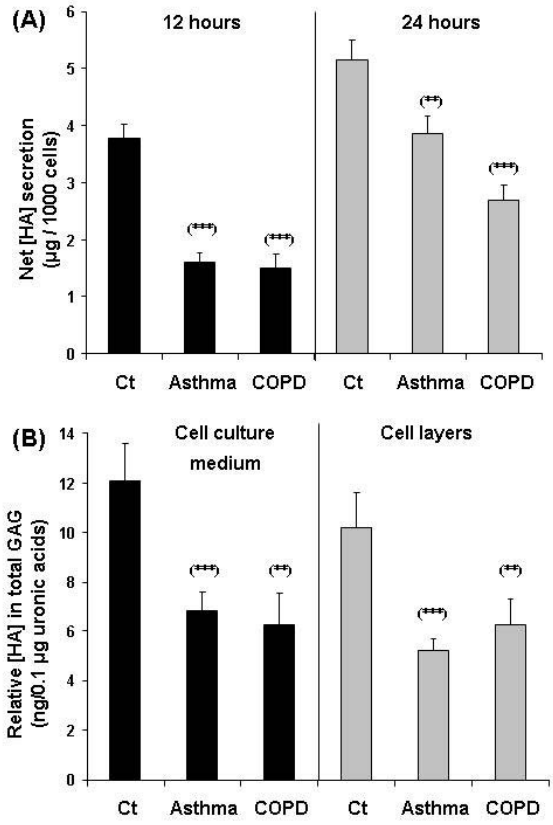

Figure 3. Disease-specific expression of HA synthases (HAS) by ASMC. (A) Representative mRNA expression of *Has1*, *Has2*, *Has3* and *β-actin* in ASMC stimulated by 5% FSC over 24 h. Densitometric ratios of: (B) *Has1/β-actin)*, (C) *Has2/β-actin* and (D) *Has3/β-actin*. (E) Representative HAS1 and HAS2 protein levels investigated by Western blotting. GAPDH served as a loading control. Each bar represents the mean ± SEM of triplicate determinations of the *Has/β-actin* ratio calculated from ASMC established from 10 healthy donors, 11 asthma and 6 COPD patients. (*) = $p < 0.05$, (***) = $p < 0.001$. Statistical differences indicated are between controls and asthma or COPD at each time point.

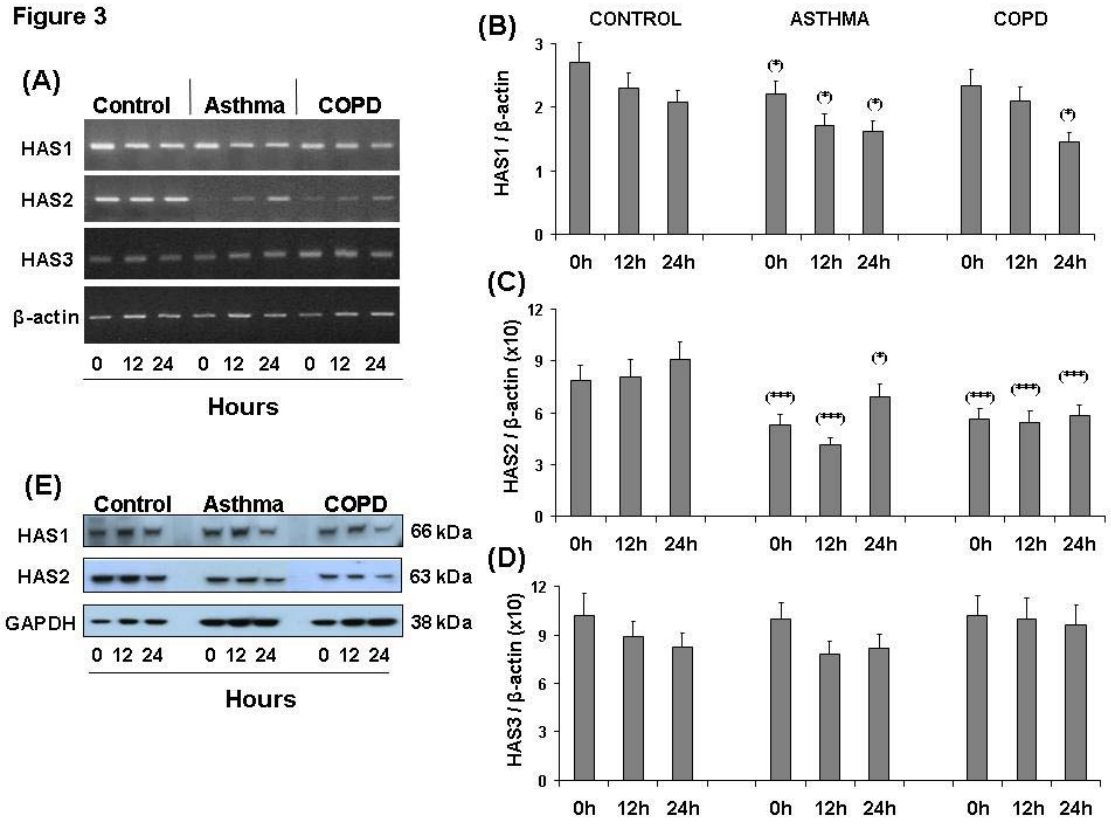

Figure 4. Disease-specific expression of Hyaluronidases (HYAL) by ASMC. (A) Representative mRNA expression of *Hyal1*, *Hyal2*, *Hyal3* and *β-actin* in ASMC stimulated with 5% FCS over 24 h. Densitometric ratios of: (B) *Hyal1/β-actin*, (C) *Hyal2/β-actin* and (D) *Hyal3/β-actin*. (E) Representative HYAL1 protein levels investigated by Western blotting. GAPDH served as a loading control. Each bar represents the mean ± SEM of triplicate determinations of the *Hyal/β-actin* ratio calculated from ASMC established from 10 healthy donors, 11 asthma and 6 COPD patients. (*) = $p < 0.05$, (***) = $p < 0.001$. Statistical differences indicated are between controls and asthma or COPD at each time point.

Figure 4

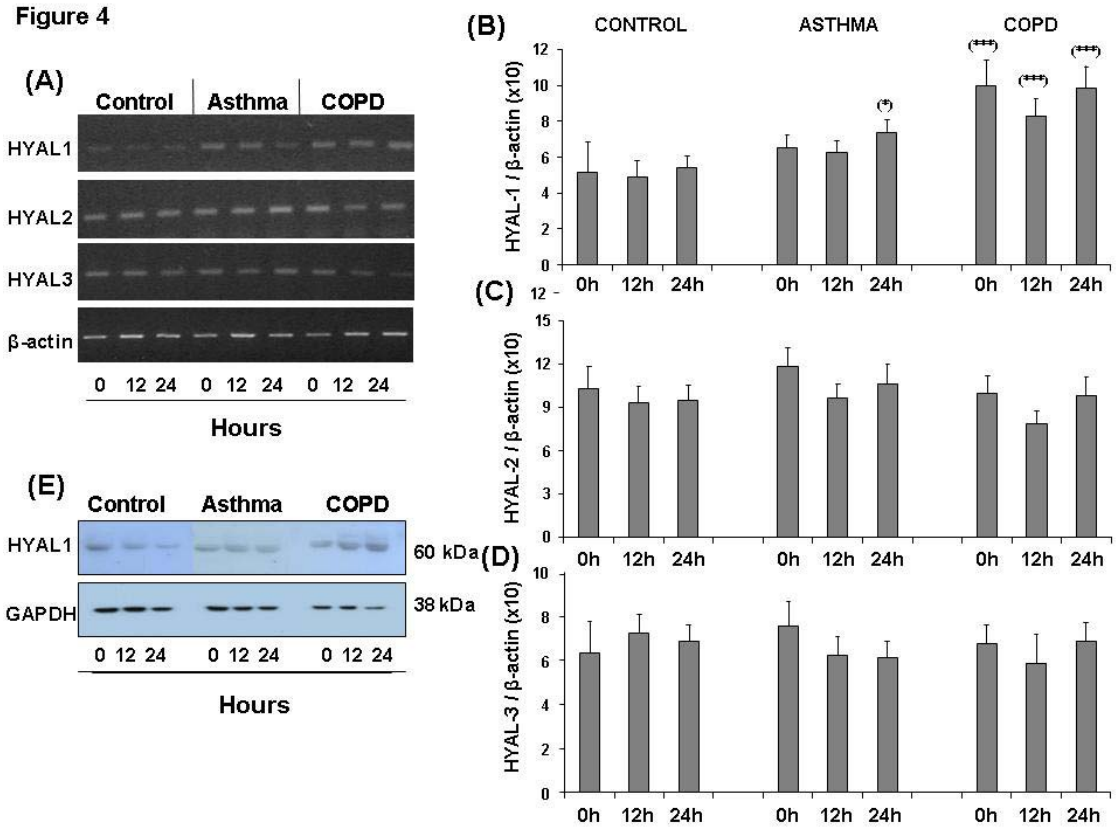

Figure 5. Determination of the molecular mass of HA by 4% PAGE. Representative results obtained after PAGE of total GAG corresponding to 4 µg of uronic acids. Gels were stained with Alcian blue, and the migration of HA, identified by hyaluronidase treatment, is indicated by arrows (right). Small arrows (left) indicate migration of GAG with known molecular mass. 1: control; 2: asthma; 3: COPD.

Figure 5

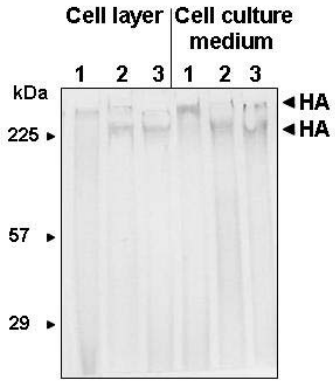

Figure 6. Disease-specific expression of HA receptors by ASMC. (A) Representative mRNA expression of *CD44*, *Rhamm* and *β-actin* in ASMC after stimulation with 5% FCS over 24 h. Densitometric ratios of: (B) *CD44/β-actin* and (C) *Rhamm/β-actin*. (D) Representative CD44 and RHAMM protein levels investigated by Western blotting. GAPDH served as a loading control. Each bar represents the mean ± SEM of triplicate determinations from ASMC of 10 donors, 11 asthma and 6 COPD patients. (*) = $p < 0.05$, (**) = $p < 0.01$, (***) = $p < 0.001$. Statistical differences indicated are between controls and asthma or COPD at each time point.

Figure 6

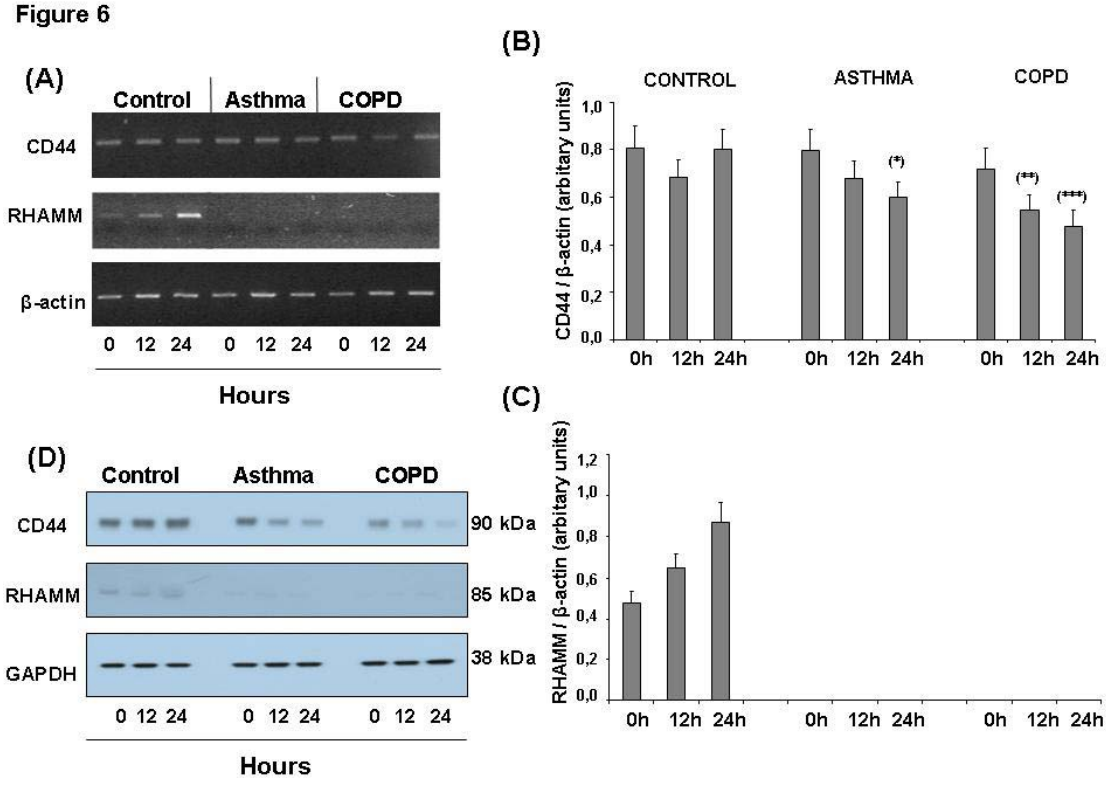
(A)
Control
Asthma
COPD
CD44
RHAMM
β-actin
0 12 24 0 12 24 0 12 24
Hours
(B)
CONTROL
ASTHMA
COPD
CD44 / β-actin (arbitary units)
1,0
0,8
0,6
0,4
0,2
0,0
(*)
(**)
(***)
0h 12h 24h 0h 12h 24h 0h 12h 24h
(C)
RHAMM / β-actin (arbitary units)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0h 12h 24h 0h 12h 24h 0h 12h 24h
(D)
Control
Asthma
COPD
CD44
RHAMM
GAPDH
90 kDa
85 kDa
38 kDa
0 12 24 0 12 24 0 12 24
Hours

Table 1

Clinical characteristics of patients

Diagnosis	Age (years)	Gender	FEV1 (% predicted)	Therapy
Healthy	28	male	100	none
Healthy	52	male	NA	none
Healthy	61	female	98	none
Healthy	43	male	NA	none
Healthy	36	male	97	none
Healthy	57	male	NA	none
Healthy	53	male	100	none
Healthy	21	female	100	none
Healthy	24	female	NA	none
Healthy	25	male	100	none
Mean ± SD	**37.5 ± 18.7**		**99.16 ± 1.33**	
COPD	52	male	62	Corticosteroids
COPD	57	male	NA	β_2-agonists + corticosteroids
COPD	56	male	58	β_2-agonists + corticosteroids
COPD/emphysema	44	female	56	β_2-agonists + corticosteroids
COPD/emphysema	48	female	60	β_2-agonists + corticosteroids
COPD/emphysema	52	male	NA	β_2-agonists + corticosteroids
Mean ± SD	**50.5 ± 4.1**		59 **± 2.58**	
Asthma	20	female	75	β_2-agonists + corticosteroids
Asthma	40	male	48	β_2-agonists + corticosteroids
Asthma	43	female	85	none
Asthma	48	male	NA	β_2-agonists + corticosteroids
Asthma	53	female	63	β_2-agonists + corticosteroids
Asthma	18	female	82	none
Asthma	33	female	NA	β_2-agonists + corticosteroids
Asthma	25	male	79	none
Asthma	64	male	NA	none
Asthma	52	male	58	β_2-agonists + corticosteroids
Asthma	33	female	NA	β_2-agonists + corticosteroids
Mean ± SD	**39 ± 23.7**		**70 ± 13.5**	

NA: not available

Table 2
Details of primers used

Primers	Sequences	Cycles	Annealing Temperature	Product size (bp)
Has1	For: GCGATACTGGGTAGCCTTCA Rev: GGTTGTACCAGGCCTCAAGA	30	57 ^{o}C	131
Has2	For: ACAGACAGGCTGAGGACGAC Rev: GCTGTGATTCCAAGGAGGAG	28	57 ^{o}C	126
Has3	For: GTCATGTACACGGCCTTCAA Rev: CCTACTTGGGGATCCTCCTC	35	59 ^{o}C	130
Hyal1	For: GTGCTGCCCTATGTCCAGAT Rev: ATTTTCCCAGCTCACCCAGA	35	59 ^{o}C	132
Hyal2	For: TCTACCATTGGCGAGAGTG Rev: AGCAGCCGTGTCAGGTAAT	27	57 ^{o}C	119
Hyal3	For: GATCTGGGAGGTTCCTGTCC Rev: AGAGCTGGAGAGGCTCAGGT	30	57 ^{o}C	110
CD44	For: ATGGACAAGTTTTGGTGGCA Rev: GTCCCAGCTCCCTGTAATGG	22	57 ^{o}C	1546
Rhamm	For: GTCACCTTCAGTTTCTGGAGCTGG Rev: GCAACATCAATAACAACAAGACGA	32	55 ^{o}C	2265
β-actin	For: ACACTGTGCCCATCTACGAGG Rev: AGGGGCCGGACTCGTCATACT	20	57 ^{o}C	621

Has1: Hyaluronic acid synthase 1, *Has2:* Hyaluronic acid synthase 2, *Has3*: Hyaluronic acid synthase 3, *Hyal1*: Hyaluronidase 1, *Hyal2*: Hyaluronidase 2, *Hyal3*: Hyaluronidase 3, *Rhamm*: receptor hyaluronic acid-mediated motility

Table 3

Enzymatic treatment with GAG-degrading enzymes of total GAGs isolated and purified from primary ASMC obtained from healthy donors and patients with asthma or COPD

Substrate	GAG population*	Chondroitinase ABC	Chondroitinase B	Hyaluronidase	Heparinase	Heparitinase	Keratanase
Control cell layer	G1	(-)	(-)	(+)	(-)	(-)	(-)
	G2	(-)	(-)	(-)	(-)	(+)	(-)
	G3	(+)	(+)	(-)	(-)	(-)	(-)
Control medium	G1	(-)	(-)	(+)	(-)	(-)	(-)
	G2	(-)	(-)	(-)	(-)	(+)	(-)
	G3	(+)	(+)	(-)	(-)	(-)	(-)
	G4	(+)	(-)	(-)	(-)	(-)	(-)
Asthma cell layer	G1	(-)	(-)	(+)	(-)	(-)	(-)
	G2	(-)	(-)	(-)	(-)	(+)	(-)
	G3	(+)	(+)	(-)	(-)	(-)	(-)
Asthma medium	G1	(-)	(-)	(+)	(-)	(-)	(-)
	G2	(-)	(-)	(-)	(-)	(+)	(-)
	G3	(+)	(+)	(-)	(-)	(-)	(-)
	G4	(+)	(-)	(-)	(-)	(-)	(-)
COPD cell layer	G1	(-)	(-)	(+)	(-)	(-)	(-)
	G2	(-)	(-)	(-)	(-)	(+)	(-)
	G3	(+)	(+)	(-)	(-)	(-)	(-)
COPD medium	G1	(-)	(-)	(+)	(-)	(-)	(-)
	G2	(-)	(-)	(-)	(-)	(+)	(-)
	G3	(+)	(+)	(-)	(-)	(-)	(-)
	G4	(+)	(-)	(-)	(-)	(-)	(-)
CSA		(+)	(-)	(-)	(-)	(-)	(-)
DS		(+)	(+)	(-)	(-)	(-)	(-)
CSC		(+)	(-)	(-)	(-)	(-)	(-)
H		(-)	(-)	(-)	(+)	(-)	(-)
HA		(-)	(-)	(+)	(-)	(-)	(-)
HS		(-)	(-)	(-)	(-)	(+)	(-)
KS		(-)	(-)	(-)	(-)	(-)	(+)

Total GAG isolated and purified from primary cultures of human ASMC obtained from healthy donors (controls, n=10) and patients with asthma (n=11) and COPD (n=6) were treated with GAG-degrading enzymes. The digestion was monitored by electrophoresis on cellulose acetate membranes. CSA, chondroitin sulfate A; DS, dermatan sulfate; CSC, chondroitin sulfate C; HA, hyaluronic acid; KS, keratan sulfate; HS, heparan sulfate; H, heparin.

* GAG populations are as obtained following cellulose acetate electrophoresis and numbers correspond to those of Figure 1B.