

Steroids and β_2 -Agonists Regulate Hyaluronan Metabolism in Asthmatic Airway Smooth Muscle Cells

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Glycosaminoglycans (GAGs), especially hyaluronic acid (HA), regulate tissue flexibility, cell motility, and inflammation. Airway smooth muscle cells (ASMCs) of patients with asthma exhibit abnormal HA metabolism, which contributes to inflammation and remodeling. Here, we investigated the effects of glucocorticoids and long-acting β_2 -agonists (LABAs) on GAG synthesis and HA metabolism by human primary ASMCs. ASMCs were isolated from airway specimens of 10 patients without asthma and 11 patients with asthma. ASMCs were incubated with glucocorticoids, LABAs, or their combination, as well as with their specific receptor antagonists. Secreted and deposited total GAGs were measured by [³H]-glucosamine incorporation. The expression of specific GAGs was determined by ELISA and electrophoresis. The expression of HA synthases (HAS), of hyaluronidases (HYALs), and of the HA receptor CD44 was determined by RT-PCR, immunoblotting in cell cultures, and immunohistochemistry in tissue sections of asthmatic lungs. In serum-activated asthmatic ASMCs, glucocorticoids and LABAs significantly inhibited the increased secretion and deposition of total GAGs, but they stimulated secreted and deposited HA of high molecular mass. This effect was attributed to increased mRNA and protein expression of HAS-1 and to the reduced expression of HYAL-1. Furthermore, drug treatment stimulated the expression of CD44 receptors in asthmatic ASMCs. These effects of the drugs were eliminated by their respective receptor inhibitors. Our findings indicate that the combination of glucocorticoids with LABAs counteracts the pathologic degradation of HA, and thereby may reduce the proinflammatory potential of asthmatic ASMCs.

Keywords: glycosaminoglycans; asthma; hyaluronan; glucocorticoids; long-acting β_2 -agonists

Airway remodeling, which is a key feature of asthma, was thought to result from chronic airway inflammation. However, recent studies in humans revealed that remodeling occurs independent of inflammation (1). Airway wall remodeling is characterized by airway smooth muscle cell (ASMC) hyperplasia and hypertrophy, thickening of the basement membrane, and increased deposition of interstitial extracellular matrix (ECM) molecules such as glycosaminoglycans (GAGs) (2). GAGs

CLINICAL RELEVANCE

This study describes a new mechanism of action for combined glucocorticoids and long-acting β_2 -agonists in asthma therapy through the regulation of hyaluronic acid turnover. The pathologic breakdown of hyaluronic acid by asthma airway smooth muscle cells is thereby counteracted. The data support the concept that hyaluronic acid is a putative target to confront pathologic remodeling in the airways of patients with asthma.

regulate the water content of ECM, cell adhesion, cell differentiation, cell proliferation, and cell migration, and they modulate the activity of growth factors and cytokines, as well as collagen fibrillogenesis (3). In the lung, GAGs alter the viscoelastic and biomechanical properties of the tissue (4). Therefore, alterations in GAG content, synthesis, and tissue distribution play an essential role during lung development as well as in pathological processes.

Hyaluronic acid (HA) is abundant in the human lung, localized mainly in the peribronchial and interalveolar/perialveolar tissue. The molecular mass of HA determines its biological function. High-molecular-weight HA (hmw-HA) exhibits antiangiogenic, anti-inflammatory, and immune-suppressive effects, whereas low-molecular-weight HA (lmw-HA) is proangiogenic and proinflammatory (5).

The polymerization of HA is regulated by the action of three HA synthases (HAS 1–3) (6). HA is metabolized by hyaluronidases (HYALs), which are present in various tissues, including the lung (7). The effects of HA are mediated through its main receptor CD44 (8), but also by the receptor for HA-mediated motility (9).

We recently showed that, in asthmatic ASMCs, there is a decreased expression of HA associated with the down-regulation of HAS-1, the up-regulation of HYAL-1, and the decreased expression of CD44 receptors (10). In this study, we investigated the effects of glucocorticoids and long-acting β_2 -agonists (LABAs), which comprise the cornerstone treatment for asthma, on the expression of HA, its metabolizing enzymes, and its receptors in human primary ASMCs from patients without asthma and patients with asthma. We report that drug treatment induced the secretion and deposition of hmw-HA, and this was associated with the increased expression of HAS-1, the decreased expression of HYAL-1, and the increased expression of CD44, indicating that glucocorticoids and LABAs reverse pathological HA metabolism in asthmatic ASMCs.

MATERIALS AND METHODS

Cell Cultures

With the approval of the Ethics Committee at University Hospital Basel, primary ASMCs were isolated from dissected airway muscle

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bundles obtained from the endobronchial biopsies of 10 nonasthmatic volunteers and 11 patients with mild to moderate asthma (11). The patients did not manifest exacerbations, and received no medication 1 week before their bronchoscopy. Written, informed consent was obtained from each patient. The clinical characteristics of the patients are described in Table E1 in the online supplement.

ASMCs were characterized by positive immunostaining for α -smooth muscle actin and calponin, whereas they stained negative for fibronectin (12). Confluent ASMCs were serum-deprived (0.1% FCS) for 24 hours before treatment with budesonide, formoterol, fluticasone, and salmeterol (Sigma Chemical Co., St. Louis, MO) (13). In certain experiments, ASMCs were preincubated for 30 minutes with the glucocorticoid receptor antagonist RU458 (Calbiochem, Lucerne, Switzerland) and/or the β_2 -adrenergic receptor antagonist propranolol (Calbiochem), before the addition of the drugs.

[³H]-Glucosamine Incorporation

To measure *de novo* GAG synthesis, confluent ASMCs were incubated with medium containing either 0.1% or 5% FCS in the presence of 0.5 μ Ci/ml [³H]-glucosamine (GE Healthcare Europe GmbH, Glattbrugg, Switzerland) for 24 hours. The incorporation of [³H]-glucosamine into GAGs was measured as previously described (10).

Isolation, Fractionation, and Characterization of GAGs

GAGs were isolated and purified from the culture media and the cell layers (10). The uronic acid content of total GAGs was measured colorimetrically. The fractionation of GAGs was achieved by electrophoresis on cellulose acetate membranes. The characterization of individual GAGs was achieved by treatment with GAG-degrading enzymes (see Table E2 in the online supplement). The molecular mass of GAGs was analyzed by electrophoresis on agarose gels (14).

Measurements of HA

The relative amounts of HA in total GAGs were measured in aliquots containing 0.1 μ g of uronic acid, and the amounts of HA secreted by ASMCs were measured in the supernatants by ELISA (Corgenix, Westminster, CO).

Quantitative RT-PCR

Total RNA was extracted using the Qiagen (Hilden, Germany) extraction kit (15). The primers were designed by Primer Express software (version 2.0; Applied Biosystems, Foster City, CA) (7).

Western Blot Analysis

Total protein extracts were prepared from confluent ASMCs, and were quantified according to the Bradford assay (Bio-Rad, Glattbrugg, Switzerland). Ten micrograms of proteins were applied to electrophoresis on 4–15% SDS-PAGE, and Western blotting was performed as previously described (10).

Immunohistochemistry

Paraffin-embedded human bronchial biopsies from six patients with asthma were stained with biotinylated HA-binding protein (HABP; Seigakaku, Tokyo, Japan), HYAL-1, HAS-1, and CD44 antibodies (Novus Biologicals, Littleton, CO), using the Histostain Plus Kit (Zymed, San Francisco, CA) (7).

Statistical Analysis

The computer software SPSS, version 16.0 (SPSS, Inc., Chicago, IL), was used. The normal distribution of data was checked using Kolmogorov–Smirnov analysis. Parametric data were analyzed according to a Student nonpaired *t* test. Two-tailed levels of significance were used in all statistical calculations. Differences were considered to be significant at **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

RESULTS

Effects of Glucocorticoids and LABAs on Total GAG Secretion and Deposition by ASMCs

In each set of experiments, a preliminary investigation was performed, which indicated that each drug administered at concentrations 10^{-9} to 10^{-6} M produced a dose–response effect, with maximum effect achieved at 10^{-7} M and 10^{-6} M. For this reason, a submaximal dose of 10^{-8} M was routinely used.

Secretion of total GAGs. Total GAG synthesis was assessed by [³H]-glucosamine incorporation. When cells were grown in the presence of 0.1% FCS, [³H]-glucosamine incorporation in secreted GAGs constituted $1,820 \pm 73$ counts per minute (cpm) (mean $\pm$ SD) in asthmatic ASMCs, and $2,255 \pm 113$ cpm (mean $\pm$ SD) in nonasthmatic ASMCs (i.e., a nonsignificant difference). Budesonide significantly reduced total GAG secretion by 12% (*P* < 0.01) in nonasthmatic ASMCs, and by 17% (*P* < 0.01) in asthmatic ASMCs (Figure 1A). No significant difference in the effect of budesonide was evident between asthmatic and nonasthmatic cells. Formoterol did not alter GAG secretion in nonasthmatic or asthmatic ASMCs (Figure 1A). However, the combination of formoterol and budesonide significantly reduced total GAG secretion by 11% (*P* < 0.05) only in asthmatic ASMCs (Figure 1A). No significant difference was evident in the effect of the combination of drugs between asthmatic and nonasthmatic cells.

When cells were grown in the presence of 5% FCS, [³H]-glucosamine incorporation in secreted GAGs constituted $2,310 \pm 100$ cpm (mean $\pm$ SD) in asthmatic ASMCs, and $3,240 \pm 149$ cpm (mean $\pm$ SD) in nonasthmatic ASMCs (statistically significant, at *P* < 0.05). Budesonide, formoterol, and their combination exerted no significant effect on total GAG secretion by nonasthmatic ASMCs (Figure 1B). However, in asthmatic ASMCs, budesonide, formoterol, and their combination significantly reduced total GAG secretion by 28% (*P* < 0.001), 10% (*P* < 0.01), and 18% (*P* < 0.01), respectively (Figure 1B). No significant differences in the effects of the drugs alone or their combination between asthmatic and nonasthmatic cells were evident.

Deposition of total GAGs. In the presence of 0.1% FCS, [³H]-glucosamine incorporation in deposited GAGs constituted 835 ± 33 cpm (mean $\pm$ SD) in asthmatic ASMCs, and 890 ± 50 cpm (mean $\pm$ SD) in nonasthmatic ASMCs (i.e., a nonsignificant difference). Budesonide alone significantly reduced GAG deposition in nonasthmatic ASMCs by 15% (*P* < 0.01), and this effect was increased when combined with formoterol (30% decrease, *P* < 0.01), which alone exerted no significant effect (Figure 1A). In asthmatic ASMCs, budesonide significantly reduced GAG deposition by 16% (*P* < 0.01) (Figure 1A). Formoterol exerted no significant effect. However, its combination with budesonide decreased total GAG deposition by 20% (*P* < 0.01) (Figure 1D). No significant differences in the effects of the drugs alone or their combination between asthmatic and nonasthmatic cells were evident.

In the presence of 5% FCS, [³H]-glucosamine incorporation in deposited GAGs constituted 950 ± 90 cpm (mean $\pm$ SD) in asthmatic ASMCs, and $1,090 \pm 66$ cpm (mean $\pm$ SD) in nonasthmatic ASMCs (i.e., a nonsignificant difference). Budesonide and formoterol alone did not alter total GAG deposition in nonasthmatic or asthmatic ASMCs. However, the combination of these drugs significantly reduced GAG deposition by 12% (*P* < 0.05) in nonasthmatic ASMCs, and by 13% (*P* < 0.01) in asthmatic ASMCs (Figure 1B). Similar results were obtained with fluticasone and salmeterol (data not shown). No significant differences in the effects of the drugs alone or their combination between asthmatic and nonasthmatic cells were evident.

These results indicate an inhibitory effect of LABAs and glucocorticoids on total GAG secretion and deposition by ASMCs.

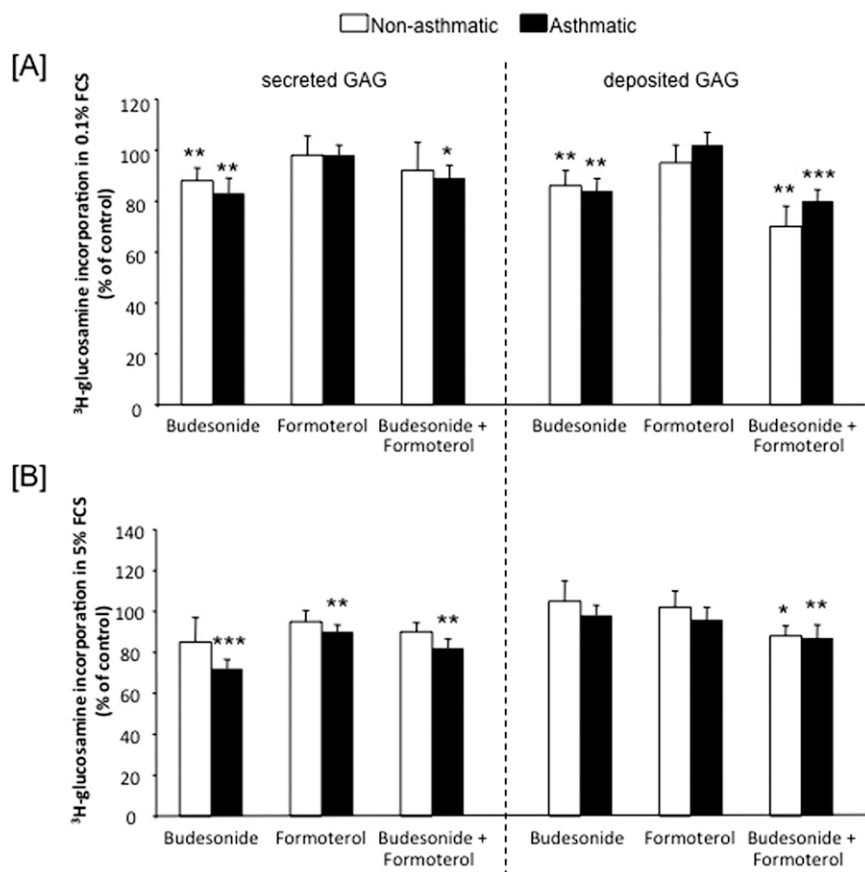


Figure 1. Budesonide and formoterol alter total glycosaminoglycan (GAG) secretion and deposition by primary airway smooth muscle cells (ASMCs) from nonasthmatic and asthmatic patients. Confluent primary ASMCs were incubated for 24 hours with (A) 0.1% FCS or (B) 5% FCS in the presence of [^3H]-glucosamine (0.5 $\mu\text{Ci}/\text{ml}$) and 10^{-8} M of budesonide, formoterol, or their combination. Total GAG secretion and deposition in the extracellular matrix (ECM) were determined as counts per minute (cpm) and expressed as a percentage of control values (incubation in the absence of drugs). Experiments were performed in triplicate for each patient. Bars represent the means $\pm$ SEMs of 10 patients without asthma and 11 patients with asthma. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Effects of Glucocorticoids and LABAs on Individual GAGs

Secretion of individual GAGs. In nonasthmatic and asthmatic ASMCs, the fractionation of secreted GAGs revealed four distinct GAG populations, designated as G1, G2, G3, and G4, which migrated with the same mobility as HA, heparan sulfate (HS), dermatan sulfate (DS), and chondroitin sulfate (CS), respectively (Figures 2A and 2B). Enzymatic characterization with specific GAG-degrading enzymes (Table E2) confirmed that G1 is HA, G2 is HS, G3 is DS, and G4 is CS. The same GAG was secreted by nonasthmatic and asthmatic ASMCs. Neither budesonide nor formoterol, nor their combination, altered the type of GAG secreted by nonasthmatic and asthmatic ASMCs (Figures 2A and 2B).

However, from the intensity of Alcian blue staining, which is relative to the amount of GAGs, disease-specific differences in the amounts of individual GAGs were indicated, which confirmed our earlier studies (10). Thus, HA secretion was significantly decreased in the cell culture medium of asthmatic ASMCs, compared with nonasthmatic control cells (Figures 2A and 2B). Budesonide, formoterol, and their combination did not alter the relative amounts of individual GAGs secreted by nonasthmatic ASMCs (Figure 2A). Furthermore, in asthmatic ASMCs, neither budesonide nor formoterol alone affected the amount of individual GAG secretion (Figure 2B). However, their combination increased HA secretion (Figure 2B). Similar results were also obtained with fluticasone and salmeterol (data not shown).

Deposition of individual GAGs. The fractionation of deposited GAGs revealed only three distinct GAG populations, designated as G1, G2, and G3 (Figures 2C and 2D), which migrated with the same mobility as HA, HS, and DS, respectively. Enzymatic characterization with specific GAG-degrading enzymes (Table E2) confirmed that G1 is HA, G2 is HS, and G3 is

DS. No differences were evident in the types of GAG deposited by ASMCs from patients without asthma and patients with asthma. Furthermore, budesonide, formoterol, and their combination did not alter the type of GAG that was deposited in the cell layers by either group of ASMCs (Figures 2C and 2D).

However, the intensity of Alcian blue staining confirmed the decreased amount of HA deposited by asthmatic ASMCs compared with nonasthmatic cells (10) (Figures 1C and 2D). Budesonide, formoterol, and their combination did not affect the relative amounts of deposited GAGs by nonasthmatic ASMCs (Figures 1C and 2D). However, budesonide, formoterol, and their combination increased HA deposition by asthmatic ASMCs (Figure 2D). Similar results were also obtained with fluticasone and salmeterol (data not shown).

Effects of Glucocorticoids and LABAs on HA Secretion and Deposition by ASMCs

To quantify the effects of budesonide, formoterol, and their combination on the HA secretion and deposition by ASMCs shown in Figure 2, we assessed the HA content in total GAGs by ELISA. The relative amount of HA in total secreted GAGs (0.1 μg of uronic acids) was 5.9 ± 0.9 ng (mean $\pm$ SD) for asthmatic ASMCs, and 12.2 ± 1.6 ng (mean $\pm$ SD) for nonasthmatic ASMCs, indicating a statistically significant ($P < 0.01$) reduction in the relative amount of secreted HA in asthma. The relative amount of HA in total deposited GAGs (0.1 μg of uronic acids) was 4.3 ± 0.5 ng (mean $\pm$ SD) for asthmatic ASMCs and 10.1 ± 1.3 ng (mean $\pm$ SD) for nonasthmatic ASMCs, indicating a statistically significant ($P < 0.01$) reduction in the relative amount of HA deposition in asthma.

In nonasthmatic ASMCs, neither of the drugs alone, nor their combination, altered the relative HA content in secreted or deposited GAGs (Figure 3A). In asthmatic ASMCs, budesonide or formoterol alone exerted no significant effect, but their

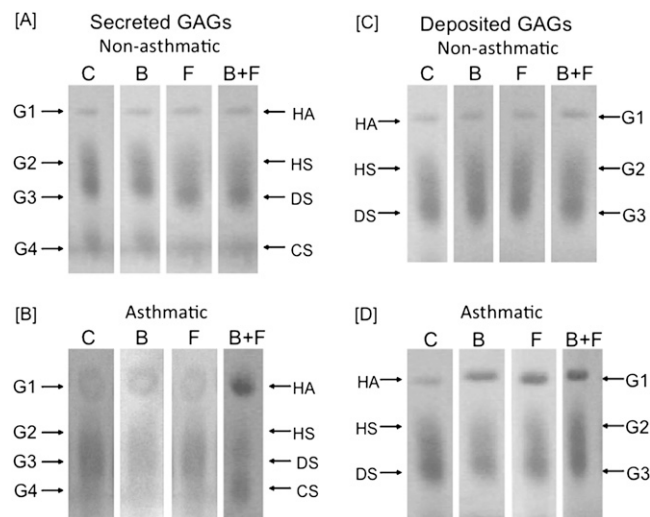


Figure 2. Budesonide and formoterol alter the amount of individual GAGs secreted and deposited by the ASMCs of patients without asthma and patients with asthma. Representative analyses are shown of electrophoretic mobility on the cellulose acetate membranes of 4 μ g of total GAGs (G1–G4) isolated and purified from the primary ASMCs of 10 patients without asthma and 11 patients with asthma. Total GAGs were isolated and purified from cell culture medium (secreted GAGs) or cell layers (deposited GAGs), 24 hours after stimulation with 5% FCS in the absence (C) or in the presence of 10^{-8} M budesonide (B), formoterol (F), or their combination (B + F). The migration of commercially available markers is indicated by arrows. HA, hyaluronic acid; HS, heparan sulfate; DS, dermatan sulfate; CS, chondroitin sulfate.

combination significantly induced relative HA secretion by 42% ($P < 0.05$) (Figure 3A). This effect of the combination of drugs was significantly higher in asthmatic ASMCs compared with nonasthmatic cells ($P < 0.01$). Furthermore, in asthmatic ASMCs, budesonide significantly increased the relative amount of HA in deposited GAGs by 118% ($P < 0.001$) (Figure 3A). Formoterol also significantly increased the relative amount of HA in deposited GAGs by 69% ($P < 0.05$), and the combination of the two drugs exerted an additive effect (192% increase; $P < 0.001$) (Figure 3A). The effect of each drug alone, or of their combination, on the relative amount of HA in deposited GAGs was significantly higher in asthmatic compared with nonasthmatic ASMCs ($P < 0.001$ for budesonide, $P < 0.05$ for formoterol, and $P < 0.001$ for their combination) (Figure 3A).

The net secretion of HA by ASMCs was also measured in aliquots of the cell culture media, 24 hours after drug treatment, by ELISA. Asthmatic ASMCs secreted significantly less HA than nonasthmatic ASMCs (3.8 μ g HA per 1,000 asthmatic ASMCs, and 5.1 μ g HA per 1,000 nonasthmatic ASMCs; $P < 0.01$). Budesonide or formoterol alone exerted no effect on HA secretion by nonasthmatic or asthmatic ASMCs (Figure 3B). However, the combination of drugs significantly induced the secretion of HA by 47% ($P < 0.01$) in nonasthmatic ASMCs, and by 80% ($P < 0.001$) in asthmatic ASMCs (Figure 3B). The effect of the combination of drugs on HA secretion was significantly higher in asthmatic versus nonasthmatic ASMCs ($P < 0.05$). Similar results were obtained with fluticasone and salmeterol (data not shown).

We further investigated the effects of the combination of glucocorticoids and LABAs on the size of HA secreted and deposited by nonasthmatic and asthmatic ASMCs, using agarose gel electrophoresis (Figure 3C). GAGs were stained with Stains-All (Sigma-Aldrich), and migrated on the gels as smears of various molecular masses. The migration of HA on agarose gels was identified in duplicate samples treated with hyaluronidase

before electrophoresis and the absence of staining from agarose gels (data not shown). Nonasthmatic ASMCs secreted and deposited HA of high molecular masses (greater than 1,000 kD) (Figure 3C, lanes 4 and 5). Asthmatic ASMCs secreted HA of an average molecular size of 250 kD, and deposited HA of diverse molecular sizes from 65 to greater than 1,000 kD (Figure 3C, lanes 6). The treatment of cells with budesonide and formoterol resulted in the secretion and deposition of hmw-HA, estimated to be greater than 1,000 kD (Figure 3C, lanes 7).

Effects of Glucocorticoids and LABAs on HA-Metabolizing Enzymes

To delineate the effects of the drugs on HA homeostasis, we investigated the expression of HA-metabolizing enzymes after 24 hours of incubation. HAS-1 and HAS-2 gene expression was significantly reduced in asthmatic ASMCs, compared with nonasthmatic ASMCs, by 22.5% ($P < 0.05$) and 24% ($P < 0.05$), respectively.

Budesonide alone exerted no effect on HAS-1 gene expression in nonasthmatic ASMCs (Figure 4A). However, it significantly increased HAS-1 gene expression in asthmatic ASMCs by 50% ($P < 0.05$). Formoterol significantly induced HAS-1 gene expression by 60% ($P < 0.05$) and by 100% ($P < 0.01$) in nonasthmatic and asthmatic ASMCs, respectively (Figure 4A). This effect of formoterol was significantly higher in asthmatic compared with nonasthmatic ASMCs ($P < 0.05$). The combination of these drugs exerted an additive stimulatory effect on HAS-1 gene expression by 80% ($P < 0.01$) and by 150% ($P < 0.01$) in nonasthmatic and asthmatic ASMCs, respectively (Figure 4A). This effect of the drug combination was significantly higher in asthmatic ASMCs compared with nonasthmatic ASMCs ($P < 0.01$). Similar effects were also observed after 12 hours and 48 hours (data not shown).

RU458, an antagonist of glucocorticoid receptors, inhibited the stimulatory effect of budesonide (used alone or in combination) on HAS-1 gene expression (Figure 4A). Propranolol, an antagonist of adrenergic receptors, inhibited the stimulatory effect of formoterol (used alone or in combination) on HAS-1 gene expression (Figure 4A). These results indicate that the effects of these drugs on HAS-1 gene expression, as already described, are mediated by glucocorticoid and β_2 -adrenergic receptors.

Western blot experiments for HAS-1 confirmed the results at the protein level we have already described. HAS-1 protein expression was stimulated by formoterol and its combination with budesonide in nonasthmatic and in asthmatic ASMCs (Figure 4B).

None of the drugs, alone or in combination, affected HAS-2 gene expression in nonasthmatic or asthmatic ASMCs (Figure 4C).

Regarding the gene expression of hyaluronidases, a significant increase was evident in HYAL-1 gene expression by 35% ($P < 0.05$) in asthmatic ASMCs, compared with nonasthmatic ASMCs. Budesonide significantly inhibited HYAL-1 gene expression by 40% ($P < 0.01$) in nonasthmatic ASMCs, and by 60% in asthmatic ASMCs ($P < 0.001$) (Figure 5A). This effect of budesonide was significantly higher in asthmatic ASMCs compared with nonasthmatic ASMCs ($P < 0.001$). Formoterol exerted no effect on HYAL-1 gene expression in nonasthmatic cells. However, it significantly inhibited HYAL-1 gene expression in asthmatic cells by 40% ($P < 0.05$) (Figure 5A). The combination of budesonide and formoterol exerted an additive inhibitory effect on HYAL-1 gene expression by 30% ($P < 0.05$) in nonasthmatic ASMCs, and by 50% ($P < 0.001$) in asthmatic ASMCs (Figure 5A). The effect of the drugs in combination was significantly greater in asthmatic ASMCs, compared with nonasthmatic ASMCs ($P < 0.001$). Similar effects were observed at 12 hours and after 48 hours (data not shown).

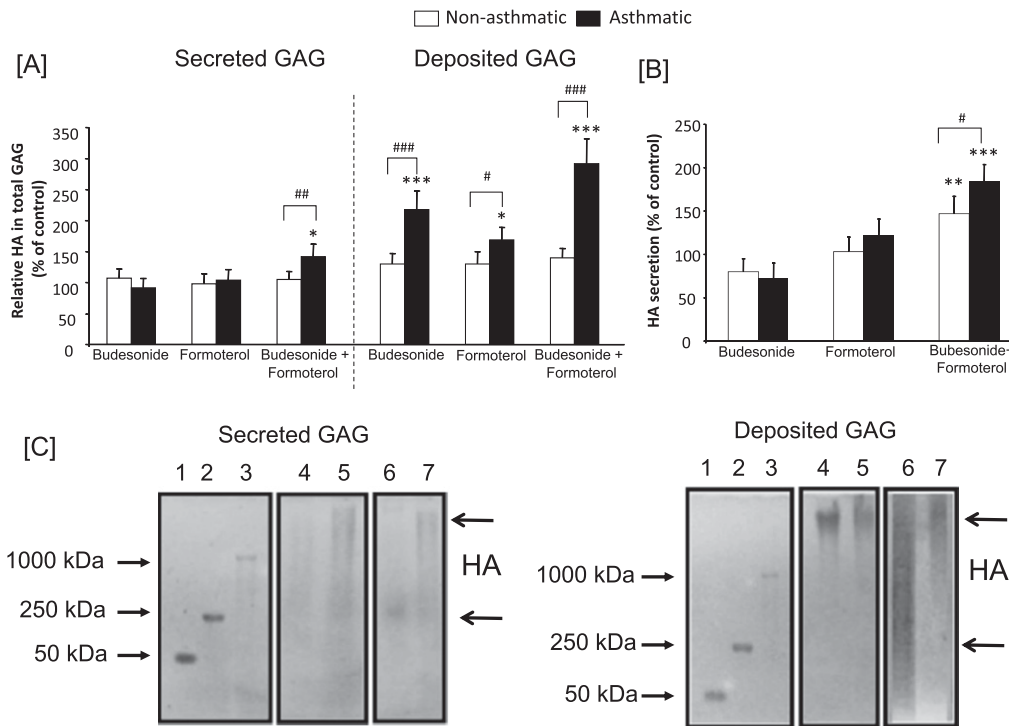


Figure 3. Budesonide and formoterol alter HA secretion and deposition by ASMCs. (A) Total GAGs were isolated and purified from cell culture medium (secreted GAGs) or cell layers (deposited GAGs), 24 hours after stimulation with 5% FCS in the absence (control) or in the presence of 10⁻⁸ M budesonide, formoterol, or their combination. The relative amount of HA was determined by ELISA in aliquots of total GAGs containing 0.1 µg of uronic acids. (B) Primary ASMCs from nonasthmatic and asthmatic patients were incubated for 24 hours with 5% FCS in the absence (control) or in the presence of 10⁻⁸ M budesonide, formoterol, or their combination. The amount of secreted HA was measured by ELISA in aliquots of cell culture medium. All results are expressed as percentages of control values. Determinations were performed in duplicate for each patient. Bars represent means ± SEM of 10 patients without asthma and 11 patients with asthma. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, for comparisons within groups. #*P* < 0.05, ##*P* < 0.01, and ###*P* < 0.001, for comparisons between different groups.

(C) Representative results were obtained after electrophoresis of total secreted or deposited GAGs (8 µg of uronic acids) on 0.8% agarose gels. Lanes 1–3, HA (1 µg) of known molecular mass; lanes 4, control subjects without asthma; lanes 5, patients without asthma treated with 10⁻⁸ M budesonide and formoterol; lanes 6, control subjects with asthma; lanes 7, subjects with asthma treated with 10⁻⁸ M budesonide and formoterol. Gels were stained overnight in the dark with a solution of 0.005% (wt/vol) Stains-All (Sigma-Aldrich) dissolved in 50% (vol/vol) ethanol and destained in H₂O. The distribution of HA on the gel was identified by hyaluronidase treatment, and is indicated by arrows.

RU458 reversed the inhibitory effect of budesonide and its combination with formoterol on HYAL-1 gene expression (Figure 5A). Furthermore, propranolol reversed the inhibitory effect of formoterol and its combination with budesonide on HYAL-1 gene expression (Figure 5A). These results indicate that the effects of drugs on HYAL-1 gene expression that we have already described are mediated by glucocorticoid and β₂-adrenergic receptors.

These results were confirmed at the protein level by immunoblot analysis. In nonasthmatic ASMCs, HYAL-1 protein expression was decreased by budesonide and its combination with formoterol. Furthermore, in asthmatic ASMCs, the inhibition of HYAL-1 protein was observed after treatment with budesonide, formoterol, or the combination of the drugs (Figure 5B).

None of the drugs, alone or in combination, exerted an effect on HYAL-2 gene expression by nonasthmatic and asthmatic ASMCs (Figure 5C).

These results indicate a more significant effect of glucocorticoids and LABAs on HA-metabolizing enzymes through gene regulation in asthmatic ASMCs compared with nonasthmatic ASMCs.

Effects of Glucocorticoids and LABAs on HA Receptors

Because most of the effects of HA are mediated via CD44 receptors, we sought to investigate the effects of drug treatment on the gene and protein expression of CD44. A significant decline of CD44 gene expression by 25% (*P* < 0.05) in asthmatic ASMCs was evident, compared with nonasthmatic ASMCs. Budesonide alone exerted no effect on CD44 gene expression in nonasthmatic ASMCs (Figure 6A). However, it significantly increased CD44 gene expression in asthmatic ASMCs by 50% (*P* < 0.01). Formoterol significantly induced CD44 gene expression by 60% (*P* < 0.01) in nonasthmatic ASMCs, and by 120% (*P* < 0.001) in

asthmatic ASMCs (Figure 6A). This effect of formoterol was significantly greater in asthmatic compared with nonasthmatic ASMCs (*P* < 0.001). The combination of drugs exerted an additive stimulatory effect on CD44 gene expression by 80% (*P* < 0.001) in nonasthmatic ASMCs, and by 260% (*P* < 0.001) in asthmatic ASMCs (Figure 6A). This effect of the drug combination was significantly greater in asthmatic compared with nonasthmatic ASMCs (*P* < 0.001). Similar effects were also observed after 12 hours and 48 hours (data not shown).

RU458 inhibited the stimulatory effect of budesonide alone and of its combination with formoterol on CD44 gene expression (Figure 6A). Propranolol inhibited the stimulatory effect of formoterol and of its combination with budesonide on CD44 gene expression (Figure 6A). These results indicate that the effects of the drugs on CD44 gene expression are mediated by glucocorticoids and β₂ adrenergic receptors.

Western blot experiments for CD44 confirmed these results at the protein level. In nonasthmatic ASMCs, CD44 protein expression was stimulated by formoterol and its combination with budesonide (Figure 6B). In asthmatic ASMCs, CD44 protein expression was stimulated by budesonide, formoterol, and their combination (Figure 6B).

These results indicate a more significant effect of glucocorticoids and LABAs in up-regulating HA receptors in asthmatic compared with nonasthmatic ASMCs.

Localization of HA, HAS-1, and HYAL-1 in Asthmatic Bronchial Biopsies

The localization of HA, HAS-1, HYAL-1, and CD44 proteins in asthmatic bronchial biopsies was investigated by

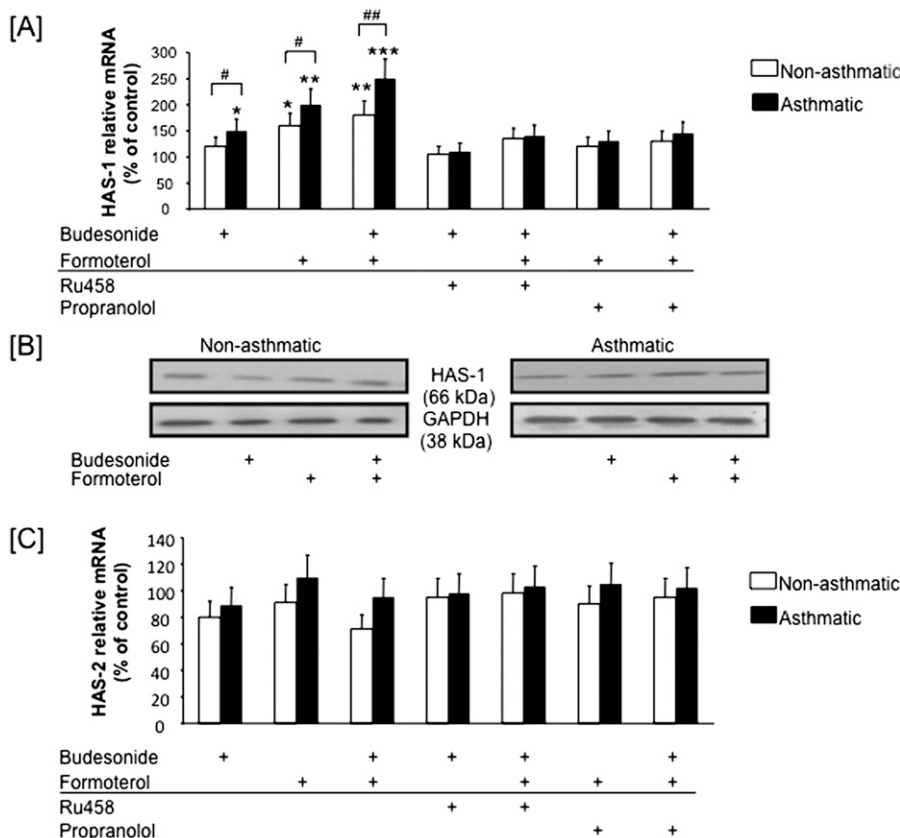


Figure 4. Budesonide and formoterol alter the gene and protein expressions of HA synthases (HAS) by ASMCs. The gene expression of HAS-1 (A) and HAS-2 (C) was investigated by quantitative RT-PCR. Data are presented as means $\pm$ SEM of the relative mRNA concentrations of 10 subjects without asthma and 11 patients with asthma as percentages over control values (incubation in the absence of drugs). (B) Representative HAS-1 protein concentrations were investigated by Western blotting. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a loading control. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, for comparisons within groups. # $P < 0.05$ and ## $P < 0.01$, for comparisons between groups.

immunohistochemistry. As shown in Figure 7A, differentiated epithelial cells and mesenchymal subepithelial cells stained positive for HA, whereas the basement membrane stained negative.

HAS-1 expression was detected in epithelial precursor cells, and even more strongly in subepithelial mesenchymal cells. Differentiated epithelial cells, mucus glands, and the basement membrane did not express HAS-1 (Figure 7B). HYAL-1 was strongly expressed by epithelial cells and mucosal gland cells, and was expressed to a lower extent by subepithelial mesenchymal cells (Figure 7C). CD44 was strongly expressed by epithelial precursor cells but not by differentiated, ciliated epithelial cells or by mesenchymal cells (Figure 7D).

DISCUSSION

In the present study, we report that the combination of glucocorticoids and LABAs reduces the deposition of total GAGs by asthmatic ASMCs, and at the same time, the drugs increase the relative content of hmw-HA in total GAGs. This observation suggests a novel mechanism by which the combination of glucocorticoids and LABAs reduces inflammation by altering the composition of the GAG content in the area of ASMC bundles in human airways.

Asthma is defined as a chronic inflammatory disorder of the large and medium sized airways, characterized by hyperresponsiveness and remodeling (16). Airway remodeling in asthma has been regarded as the result of chronic inflammation, but this interpretation has been increasingly challenged by new studies using animal models and patients with asthma. Increasing evidence suggests that remodeling is independent of inflammation, and may even precede inflammation (1).

ASMCs are thought to be involved mainly in airway narrowing, but they also play an important role in airway remodeling and inflammation in asthma (17). Constriction was considered

to be the most important function of ASMCs. However, several studies have shown that ASMCs are the source of a wide variety of proinflammatory cytokines, inflammatory mediators (18–20), and ECM molecules (10).

Asthmatic ASMCs were shown to be intrinsically different from nonasthmatic ASMCs, because they contract faster and longer, secrete more proinflammatory cytokines, and proliferate faster under inflammatory conditions (12, 21, 22). We have previously shown that the secretion and deposition of GAGs is altered in asthmatic compared with nonasthmatic ASMCs. Asthmatic ASMCs secrete and deposit significantly less HA, which is of lower molecular weight (10). The reduced production of HA by asthmatic ASMCs is related to the decreased expression of HAS-1 and HAS-2, and to the increased expression of HYAL-1 (10). The increased expression of HYAL-1 by asthmatic ASMCs is associated with the production of lmw-HA.

HA holds a unique capacity to link and retain water molecules in the ECM, and thus to hydrate tissue and control solute transport (23). Hmw-HA contributes to tissue repair (24), inhibits cell migration and chemotaxis, reduces the aggregation of polymorphonuclear leukocytes and monocytes in the airway wall and the lung (25), and prevents the elastase degradation of pulmonary elastin (26). However, during tissue injury and inflammation, an accumulation of lmw-HA occurs (27–31), which induces the expression of a variety of chemokines, cytokines, and growth factors by inflammatory cells (32–34).

In this study, we present evidence for a stimulatory effect of glucocorticoids and LABAs on HAS-1 expression by asthmatic ASMCs. HAS-1 is responsible for the synthesis of hmw-HA (2×10^5 to 2×10^6 D) (6). Furthermore, we show that the drugs reduced the expression of HYAL-1 by asthmatic ASMCs, suggesting that glucocorticoids and LABAs reverse the pathological degradation of HA in asthmatic airway walls, and favor the synthesis of a noninflammatory hmw-HA. This hypothesis is

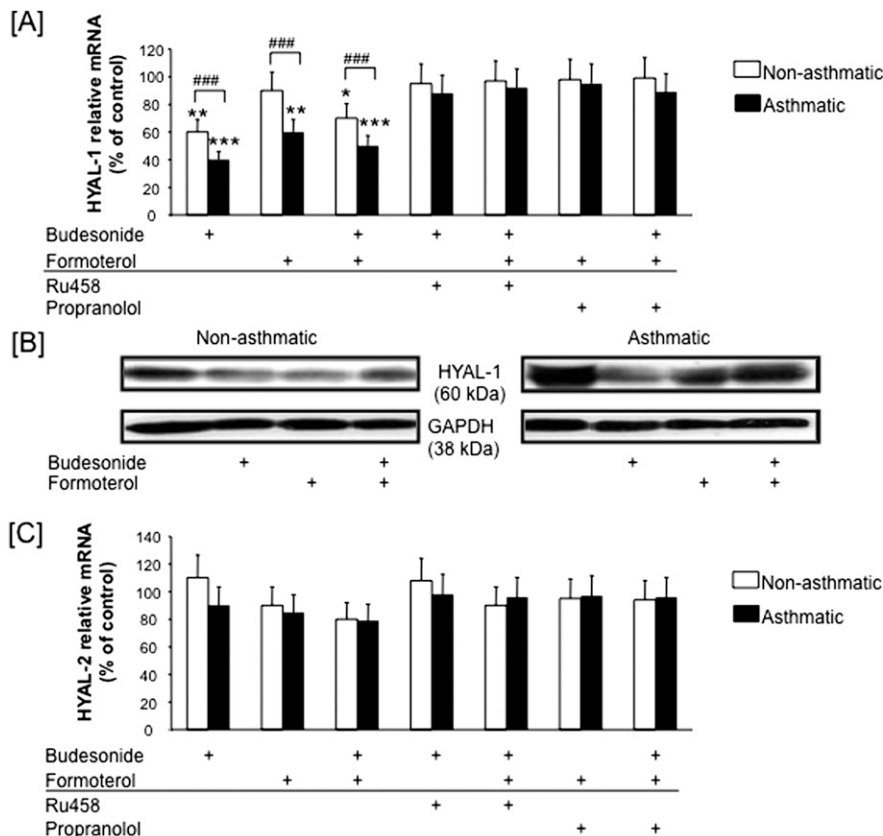


Figure 5. Budesonide and formoterol alter the gene and protein expressions of hyaluronidases (HYALs) by ASMCs. The gene expression of HYAL-1 (A) and HYAL-2 (C) was investigated by quantitative RT-PCR. Data are presented as means $\pm$ SEM of the relative mRNA concentrations of 10 patients without asthma and 11 patients with asthma, as percentages over control values (incubation in the absence of drugs). (B) Representative HYAL-1 protein concentrations were investigated by Western blotting. GAPDH served as a loading control. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, for comparisons within groups. ### $P < 0.001$, for comparisons between groups.

supported by results obtained using agarose gel electrophoresis for the determination of the molecular mass of HA. The combination of glucocorticoids and LABAs clearly shifted the poly-dispersed HA (65–1,000 kD) produced by asthmatic ASMCs to hmw-HA (above 1,000 kD). However, our data do not allow us to conclude whether this shift in molecular weight of HA was attributable to the *de novo* synthesis of hmw-HA or to the inhibition of its degradation, or to both.

We previously showed that the HA receptor CD44 is down-regulated in asthmatic ASMCs compared with nondiseased cells (10). Because CD44 is required for the clearance of HA

fragments from inflamed tissues, the reduction of CD44 in asthmatic ASMCs could lead to an impaired clearance of HA, which in turn could promote persistent inflammation and, potentially, asthma symptoms (28).

Moreover, CD44 signaling is regulated by the size of its major ligand, HA. For example, in primary vascular smooth muscle cells and fibroblasts, the binding of hmw-HA to CD44 was shown to inhibit cell proliferation, whereas the binding of lmw-HA to CD44 induces cell proliferation (8, 35). Therefore, the stimulatory effect of glucocorticoids and LABAs on the secretion and deposition of hmw-HA by asthmatic ASMCs, as well as on

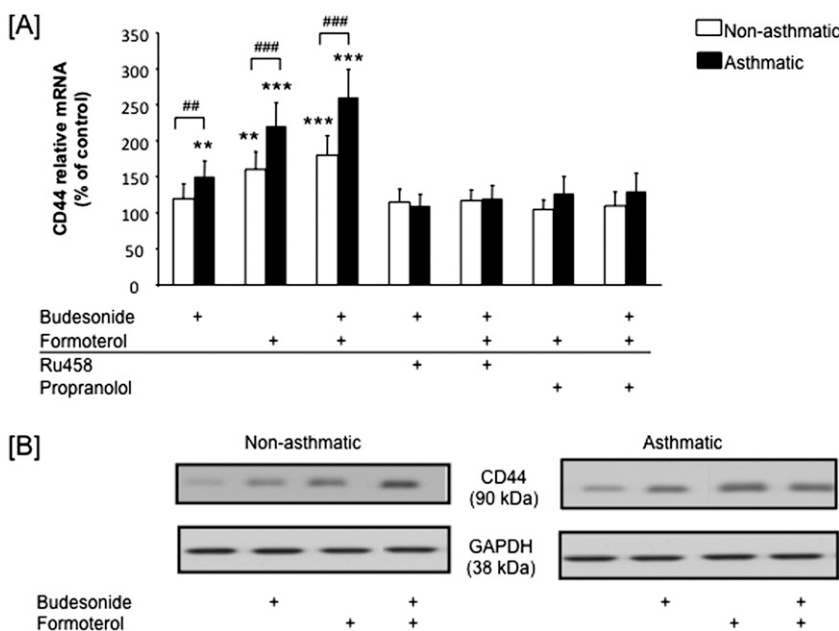


Figure 6. Budesonide and formoterol alter the gene and protein expressions of the CD44 receptor by ASMCs. (A) The gene expression of CD44 was investigated by quantitative RT-PCR. Data are presented as the means $\pm$ SEM of the relative mRNA concentrations of 10 patients without asthma and 11 patients with asthma, as percentages over control values (incubation in the absence of drugs). (B) Representative CD44 protein concentrations were investigated by Western blotting. GAPDH served as a loading control. ** $P < 0.01$ and *** $P < 0.001$, for comparisons within groups. ## $P < 0.01$ and ### $P < 0.001$, for comparisons between groups.

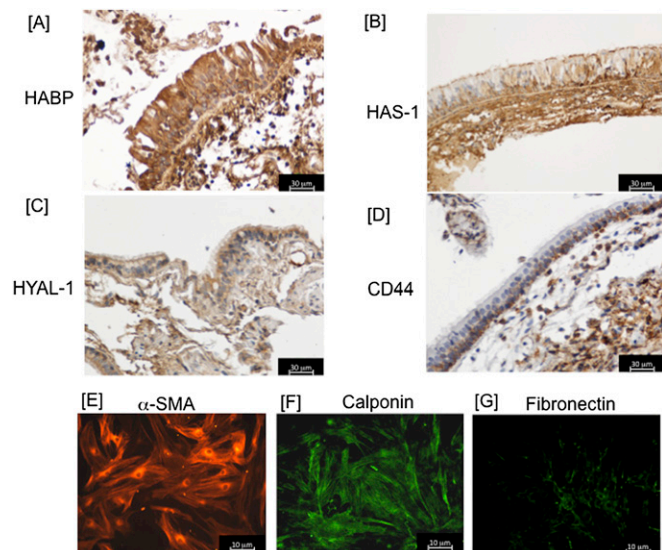


Figure 7. Immunohistochemistry. (A–D) Localization of HA using HABP, HAS-1, HYAL-1, and CD44 in human bronchial biopsies from patients with asthma. (E–G) Characterization of primary ASMCs. HA, hyaluronic acid; HABP, hyaluronic acid-binding protein; HAS, hyaluronic acid synthase; HYAL, hyaluronidase.

the expression of CD44, likely indicates the initiation of HA-mediated antimitogenic signals, with beneficial results on airway tissue remodeling.

A recent study reported that human asthmatic airway fibroblasts release significantly increased concentrations of lmw-HA compared with normal fibroblasts, suggesting that fibroblasts also contribute to the accumulation of lmw-HA in asthmatic airways (36). Furthermore, macrophages from patients with asthma show a decrease in cell-surface CD44 expression and increased responsiveness to lmw-HA stimulation, as demonstrated by increased IL-8 production (36). These data indicate that HA fragments contribute to chronic inflammation and airway remodeling in asthma.

Glucocorticoids exert a broad range of effects. However, their therapeutic action in terms of asthma is mainly related to their ability to inhibit inflammation (37). So far, no clinical or experimental evidence indicates that even long-term therapy with glucocorticoids or LABAs alone reduces airway remodeling. Several studies indicate that glucocorticoids reduce airway remodeling only when they are combined with LABAs (38–42). *In vitro*, LABAs were shown to reduce total ECM and collagen deposition by primary human lung fibroblasts, whereas the effects of glucocorticoids on ECM and collagen deposition depended on serum concentrations (13). The results presented here describe a new mechanism of glucocorticoids that leads to a beneficial modification of HA in asthmatic airways.

Our study provides new insights regarding the beneficial effects of glucocorticoids and LABAs in asthma, supporting the hypothesis that HA homeostasis is deranged in asthmatic airways, favoring the production of lmw-HA by ASMCs. Decreased CD44 expression by asthmatic ASMCs results in the impaired clearance of lmw-HA, and leads to persistent inflammation in asthmatic lungs. Treatment with glucocorticoids and LABAs moderates the production of inflammatory lmw-HA and stimulates the secretion and deposition of protective, anti-inflammatory hmw-HA and the expression of CD44, resulting in the restoration of airway tissue integrity and the alleviation of asthma symptoms.

Author disclosures are available with the text of this article at www.atsjournals.org.

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Steroids and β 2 agonists regulate hyaluronan metabolism in asthmatic airway smooth muscle cells

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ONLINE DATA SUPPLEMENT

TABLE 1**Characteristics of patients included in the study**

Non-asthmatic patients			
Age (years)	Gender	FEV1 (% predicted)	Treatment
28	male	100	none
52	male	124	none
61	female	98	none
43	male	108	none
36	male	115	none
57	male	94	none
53	male	121	none
21	female	100	none
24	female	100	none
25	male	100	none
Mean ± SEM	37.5 ± 18.7	106 ± 3.31	
Asthmatic patients			
Age (years)	Gender	FEV1 (% predicted)	Treatment (only when needed)
20	female	75	β ₂ -agonists + glucocorticoids
40	male	68	β ₂ -agonists + glucocorticoids
43	female	85	β ₂ -agonists + glucocorticoids
48	male	72	β ₂ -agonists + glucocorticoids
53	female	73	β ₂ -agonists + glucocorticoids
18	female	82	β ₂ -agonists + glucocorticoids
33	female	71	β ₂ -agonists + glucocorticoids
25	male	79	β ₂ -agonists + glucocorticoids
64	male	70	β ₂ -agonists + glucocorticoids
52	male	68	β ₂ -agonists + glucocorticoids
33	female	73	β ₂ -agonists + glucocorticoids
Mean ± SEM	39 ± 23.7	74.3 ± 1.86	

FEV1: forced expiratory volume in 1 second.

Table 2

Enzymatic treatment with GAG-degrading enzymes of total GAG isolated and purified from primary ASMC obtained from non-asthmatic and asthmatic patients

Substrate	GAG*	Chondroi- tinase ABC	Chondroi- tinase B	Hyaluro- nidase	Hepari- nase	Hepari- tinase	Kerata- nase
Non-asthmatic deposited	G1	(-)	(-)	(+)	(-)	(-)	(-)
	G2	(-)	(-)	(-)	(-)	(+)	(-)
	G3	(+)	(+)	(-)	(-)	(-)	(-)
Non-asthmatic secreted	G1	(-)	(-)	(+)	(-)	(-)	(-)
	G2	(-)	(-)	(-)	(-)	(+)	(-)
	G3	(+)	(+)	(-)	(-)	(-)	(-)
	G4	(+)	(-)	(-)	(-)	(-)	(-)
Asthmatic deposited	G1	(-)	(-)	(+)	(-)	(-)	(-)
	G2	(-)	(-)	(-)	(-)	(+)	(-)
	G3	(+)	(+)	(-)	(-)	(-)	(-)
Asthmatic secreted	G1	(-)	(-)	(+)	(-)	(-)	(-)
	G2	(-)	(-)	(-)	(-)	(+)	(-)
	G3	(+)	(+)	(-)	(-)	(-)	(-)
	G4	(+)	(-)	(-)	(-)	(-)	(-)
CSA		(+)	(-)	(-)	(-)	(-)	(-)
CSB		(+)	(+)	(-)	(-)	(-)	(-)
CSC		(+)	(-)	(-)	(-)	(-)	(-)
H		(-)	(-)	(-)	(+)	(-)	(-)
HA		(-)	(-)	(+)	(-)	(-)	(-)
HS		(-)	(-)	(-)	(-)	(+)	(-)
KS		(-)	(-)	(-)	(-)	(-)	(+)

Total GAG isolated and purified from ASMC established from non-asthmatic and asthmatic patients were treated with GAG-degrading enzymes, as described under "Materials and Methods". The digestion was monitored by electrophoresis on cellulose acetate membranes. Data shown represent observations from five different cell cultures. Standard GAG (10 µg): CSA, chondroitin sulphate A (bovine trachea); CSB, chondroitin sulphate B (porcine skin); CSC, chondroitin sulphate C (shark cartilage), hyaluronic acid (bovine trachea); KS, keratan sulphate (bovine cornea); HS, heparan sulphate (bovine intestinal mucosa); H, heparin; (all from Sigma).

* GAG populations are as obtained following cellulose acetate electrophoresis and numbers correspond to those of Figure 2.