

Allergen-Induced Airway Remodeling in Brown Norway Rats

Structural and Metabolic Changes in Glycosaminoglycans

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Increased proteoglycan (PG) deposition is a feature of airway remodeling in asthma. Glycosaminoglycans (GAGs) mediate many of the biological and mechanical properties of PGs by providing docking sites through their carbohydrate chains to bioactive ligands; therefore, it is imperative to define structural and metabolic changes of GAGs in asthma. Using a Brown Norway (BN) ovalbumin (OVA)-sensitized and -challenged rat model to induce airway remodeling, we found excessive deposition of chondroitin/dermatan (CS/DS)-, heparan (HS), and keratan (KS) sulfate GAGs in the airways and bronchoalveolar lavage cells of OVA-challenged rats. Disaccharide composition of CS/DS of OVA-challenged rats was significantly different compared with saline-treated (SAL) control rats, with increased levels of 0-, 6-, and 4-sulfated disaccharides. Increases in the amount and a change in the proportion of CS/DS versus HS GAGs were noted in OVA-challenged rats. The higher content and sulfation of CS/DS disaccharides was reflected by the increased expression of xylosyltransferase-I, β 1,3-glucuronosyltransferase-I, chondroitin-4, and chondroitin-6 sulfotransferase genes and protein expression of xylosyltransferase-I and β 1,3-glucuronosyltransferase-I in OVA-challenged rats. Genes encoding the core proteins of the CS/DS and KS-containing PGs, such as versican, biglycan, decorin, and lumican, were overexpressed in OVA-challenged rats. Our results suggest that GAG biosynthetic enzymes may be involved in the altered expression of GAGs in the airways and are potential targets for inhibiting excess PG-GAG deposition and the airway remodeling process in asthma.

Keywords: airway remodeling; glycosaminoglycans; proteoglycans

Proteoglycans (PGs), which consist of a core protein with covalently attached glycosaminoglycan (GAG) side chains, can be found attached to cell membranes and in the extracellular matrix (ECM) (1). GAGs are unbranched polymers containing repeating disaccharide units of hexuronic acid alternating with a hexosamine moiety (2). GAGs in the lung are primarily composed of chondroitin sulfate/dermatan sulfate, heparan sulfate, keratan sulfate, and hyaluronic acid (3). With the exception of hyaluronic acid, all other GAG chains are sulfated and attached to a protein core and exist as interstitial matrix PGs (e.g., versican), basement membrane PGs (e.g., perlecan), small leucine-rich repeat PGs (e.g., decorin, biglycan, lumican, and fibromodulin), or cell-associated PGs (e.g., syndecan and glypican) (4).

GAGs play a critical role in determining the water content or turgor of ECMs. In addition, GAG chains are primarily responsible for the cellular and biological activities of PGs, including cell

migration, proliferation, and survival; organization of extracellular matrices; and modulation of growth factor signaling (2, 3, 5–9). The synthesis of GAGs is regulated by glycosyltransferases (GTs) that catalyze the transfer of sugar moieties from nucleotide diphosphate-activated sugars (e.g., UDP-sugars) to acceptor molecules (2). The biosynthesis of GAGs (10) starts with the transfer of xylose onto serine residues in core proteins, followed by the sequential addition of two galactoses and a glucuronic acid forming a common tetrasaccharide linkage structure (GlcUA β 1–3Gal β 1–3Gal β 1–4Xyl β 1–O–Ser) observed in major proteoglycans. The enzymes responsible for these reactions are xylosyltransferase (XT) (11), galactosyltransferase-I (12, 13), galactosyltransferase-II (14), and GlcUA transferase I (GlcAT-I) (15, 16). The linkage tetrasaccharide acts as a primer to assemble GAG chains by the alternative addition of N-acetylglucosamine or N-acetylgalactosamine residues to the common linkage structure, leading to the creation of heparin/heparan sulfate or chondroitin sulfate/dermatan sulfate, respectively. As GAG chains polymerize, they go through a variety of sulfation and epimerization reactions by the corresponding sulfotransferase (ST) and epimerase, which result in the addition of sulfate groups to different sugars and the conversion of a portion of the D-glucuronic acid residues to L-iduronic acid, resulting in the structural diversity of GAG chains (2, 10).

Disturbances in the biosynthetic pathway of ECM components may result in quantitative or qualitative changes in the deposition of ECM proteins that may contribute to the pathogenesis of several pulmonary diseases, including asthma (17, 18). Airway remodeling in asthma is characterized by hypertrophy and hyperplasia of airway smooth muscle, thickening of the epithelial basement membrane, and subepithelial fibrosis (18). Alterations in the amount and composition of extracellular matrix proteins, especially PGs, are prominent features of airway remodeling that may contribute to lung mechanical dysfunction in asthma (19). We have previously shown increased deposition of PG core proteins such as lumican, biglycan, and versican in the subepithelial layer of the airway wall in patients with asthma as compared with that in normal control subjects (20). Subsequent studies demonstrated differences in the deposition of PG core proteins in patients with moderate versus severe asthma or in fatal asthma (21, 22). Studies in animal models of airway remodeling have also documented increased PG core protein deposition as well as changes in their distribution within the layers of the airway wall (23, 24). Together, these studies underscore the importance of PG molecules in the airway remodeling process.

Given the above findings, if airway remodeling involves alterations in PG composition and content, it is important to understand how the production and deposition of GAGs are modulated during pathological remodeling of the airway wall in asthma. Little is known about the overall changes in GAG chains or about the distribution of various GAG chains within the normal and diseased airways. A better characterization of the composition, concentration, and production of GAGs should help not only in understanding the roles these molecules may play during airway

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remodeling but also in the development of novel approaches to modulate PG deposition in asthma. In this study, we investigated the biochemical characterization, immunohistochemical localization, and expression of GAG chains, with respect to their carbohydrate composition, sulfation, content, and biosynthesis, in allergen-induced airway remodeling in Brown Norway (BN) rats. Allergen-induced airway remodeling, after multiple ovalbumin (OVA) challenges, in BN rats reproduces many of the characteristics of airway wall remodeling seen in human asthma, such as increased airway smooth muscle mass, and PG core protein deposition (24–26). Therefore, we have used this animal model to study the mechanisms of alterations of GAG deposition during airway remodeling in asthma. Our results identify, for the first time, increased expression of various GAG chains, alterations in the proportion of CS and HS GAGs, and up-regulation of glycosyltransferases and sulfotransferases as previously unrecognized phenomena that may play an important role in increased PG-GAG deposition during airway remodeling in asthma.

MATERIALS AND METHODS

Study Subjects

Endobronchial biopsy specimens from a nonasthmatic subject and a subject with severe asthma were obtained at the Fonds de Recherche en Santé du Québec Respiratory Health Network Tissue Bank. Details of patient characteristics are reported elsewhere (21).

Animal Preparation

To induce allergen-induced airway remodeling (24), pathogen-free male BN rats (weight, 200–225 g) were sensitized on Day 0 by a single subcutaneous injection of 1 mg of OVA and 100 µg of aluminum hydroxide in sterile saline (0.9%); *Bordetella pertussis* (5×10^8 heat-killed bacilli) was given intraperitoneally as an adjuvant. Two weeks later, sensitized rats were challenged with aerosols of 5% OVA or SAL at 5-day intervals on Days 14, 19, and 24. Challenges were 5 minutes in duration each, with rats receiving three separate challenges in total. Two groups of animals were studied ($n = 6$ or 7 in each group): SAL (OVA-sensitized/saline-challenged) and (2) OVA (OVA-sensitized/OVA-challenged). Details of the allergen/saline administration are given in the online supplement. All animals received humane care in compliance with the Guide to the Care and Use of Experimental Animals formulated by the Canadian Council of Animal Care, and an institutional animal ethics committee of McGill University approved the animal protocol.

Budesonide Treatment

Fourteen days after OVA sensitization, budesonide (0.1 mg/kg) or vehicle (saline) was given intranasally (in 100 µl of saline) 24 hours and 1 hour before OVA challenges as previously reported (27).

Bronchoalveolar Lavage

Two days after the last challenge (Day 26), rats were killed, bronchoalveolar lavage (BAL) was performed, and cytospin slides were prepared. Additional details are given in the online supplement.

Quantitative Real Time rtPCR

To analyze the expression of glycosyltransferases, sulfotransferases, and PG core proteins mRNAs, total RNA was extracted from the lungs using RNeasy Mini kit (Qiagen, Mississauga, ON, Canada). Normalized relative quantification of mRNA expression using a standard curve, internal control sample, and S9 as housekeeping gene was accomplished by running QRT-PCR on StepOnePlus (Applied Biosystems/Life Technologies, Carlsbad, CA) with QuantiTect SYBR Green PCR Kits (Qiagen). The sequences of oligonucleotide primers used for PCR amplification are described in Table 1 (see the online supplement for more details).

Fluorophore-Assisted Carbohydrate Electrophoresis

Disaccharide composition of hyaluronan and chondroitin/dermatan sulfate was determined as described previously (28, 29).

Isolation and Quantitation of Glycosaminoglycans

Proteinase-K-digested lung tissues were used for GAG quantitation as described in the online supplement.

Extraction of Lung Proteoglycans and Immunoblotting of PG Core Proteins

Proteoglycans were extracted (30) with 4 M guanidine hydrochloride and 100 M sodium acetate (pH 6.0) containing protease inhibitors. Chondroitinase ABC (chABC)-digested PGs were run in a 10% SDS-PAGE or in a large composite gel and analyzed by immunoblotting (see the online supplement for more details).

Immunohistochemistry

Immunostaining of GAGs, glycosyltransferases, versican, and α -smooth muscle actin (SMA) were performed (29).

Statistical Analysis

All data are presented as means $\pm$ SEM of six to seven independent observations. Comparison between SAL- and OVA-challenged groups was performed by Student's *t* test for unpaired data. Statistical analyses were performed using GraphPad Prism software (version 4.0).

RESULTS

Increased Deposition of GAG Chains in OVA-Challenged Rat Lungs and in a Subject with Asthma

To study changes in the expression of GAG chains during airway remodeling in rats, we immunostained lung sections with GAG-

TABLE 1. PRIMER PAIRS USED FOR QUANTITATIVE RT-PCR

Gene	Strand	Sequence (5'–3')
Biglycan	Forward	5'-GAATGGGAGCCTGAGTTTCTGC-3'
	Reverse	5'-CCTTGGTGATGTTGTTGGAGTGC-3'
Decorin	Forward	5'-CCACTTGGACAACAACAACTC-3'
	Reverse	5'-CTGCTATGAAGGGAGGCAGAAG-3'
Versican	Forward	5'-CAACTACCACCTCACCTACAC-3'
	Reverse	5'-ACGCTGAATGAAACCATCTTTG-3'
Lumican	Forward	5'-GATCTCTCCTACAATAAGCTC-3'
	Reverse	5'-CTTACAGAAGCTCTTGACATC-3'
Xylosyltransferase-I	Forward	5'-AGAGAAGGTGGCTCGATTCTG-3'
	Reverse	5'-GGCGATTCTGACTGGGTTG-3'
β 1,3-glucuronosyltransferase-I	Forward	5'-GAAGGACTCACCACCACAG-3'
	Reverse	5'-CCACACTGAGACACCACAG-3'
Chondroitin-4-sulfotransferase	Forward	5'-TGCTGGAAGTGATGAGGATG-3'
	Reverse	5'-AGATGTCCACGCCAAAGG-3'
Chondroitin-6-sulfotransferase	Forward	5'-TCCTGTTCTTGGCATTGTG-3'
	Reverse	5'-GAGGGATCTGCTTCAGCTTG-3'

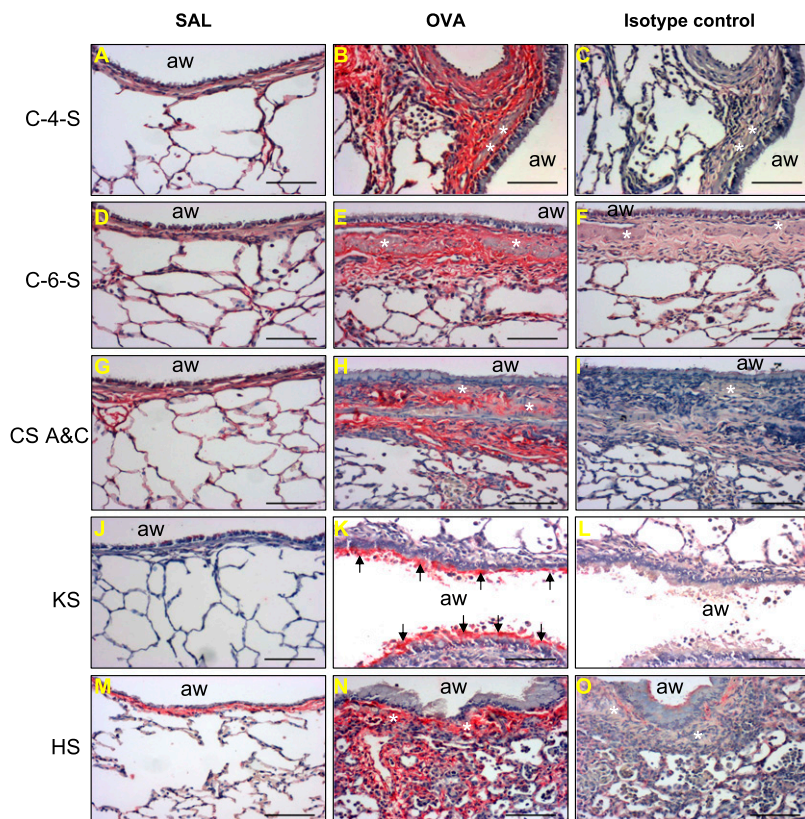


Figure 1. Immunohistochemical analysis of glycosaminoglycan (GAG) chains in the airway wall of saline (SAL)-challenged (A, D, G, J, M) and ovalbumin (OVA)-challenged (B, E, H, K, N) rats. Immunostaining of large airways for chondroitin-4-sulfate (C-4-S) (A, B), chondroitin-6-sulfate (C-6-S) (D, E), chondroitin sulfate (CS) types A and C (G, H), keratan sulfate (KS) (J, K), and heparan sulfate (HS) (M, N). Immunostaining for all GAG chains was more prominent in large airways in OVA-challenged rats compared with the SAL-challenged rats. Immunostaining was distributed in the subepithelium, basement membrane, and airway smooth muscle region for all GAGs except KS. KS staining was detected apically in the surface epithelium (closed arrows). The dark red color is indicative of strong GAG expression and deposition. C, F, I, L, O are negative controls from OVA-challenged rats stained with isotype-matched control antibody. Original magnification: $\times 200$. aw = airway lumen. Asterisks indicate airway smooth muscle layers. Scale bars = 100 μm .

specific monoclonal antibodies that identify various sulfated GAG chains: chondroitin-4-sulfate (Figures 1A–1C), chondroitin-6-sulfate (Figures 1D–1F), chondroitin sulfate types A and C (Figures 1G–1I), keratan sulfate (Figures 1J–1L), and heparan sulfate (Figures 1M–1O). In general, immunoreactivity of these GAG chains was present in the airways of the rats sensitized and repeatedly challenged with SAL. Compared with SAL-challenged rats, immunostaining of these GAG chains was highly abundant in OVA-challenged rats, which showed more intense staining in the subepithelial and adventitial layers. In addition, blood vessels demonstrated positive staining for chondroitin and heparan sulfate chains. Moreover, positive staining of these GAG chains was increased in small airways in OVA-challenged rats (data not shown). Compared with chondroitin and heparan sulfate, keratan sulfate showed distinct staining in the surface epithelium (Figure 1K, black arrows). The specificity of the immunostaining results of GAG deposition was verified by omission of the primary antibody and by incubating with isotype-matched control antibody (Figures 1D, 1F, 1I, 1L, and 1O). Furthermore, immunohistochemical analysis of GAGs was extended to human specimens. Asthmatic airways demonstrated marked immunostaining for the CS, HS GAGs, and GlcAT-I protein compared with the non-asthmatic airway (Figure 2).

BAL Cells from OVA-Challenged Rats Express More CS GAG Chains

We determined whether increased accumulation of GAG chains in the airways was associated with evidence of GAG expression in BAL cells. Immunostaining of cytospin preparations of BAL cells (Figures 3A–3F) demonstrated increased expression of chondroitin-4 (Figure 3B) and chondroitin-6 sulfate (Figure 3E) chains in OVA-challenged rats compared with SAL-challenged (Figures 3A and 3B) rats. The results also suggested that most of the immunostaining of GAG chains was intracellular; no

membrane staining could be identified. Biochemical analysis also demonstrated increased amounts of CS, DS, and KS GAGs in the BAL fluid of OVA-challenged rats compared with SAL-challenged rats (see Figure E1 in the online supplement).

Increased Expression and Colocalization of Versican, Chondroitin-4-Sulfate, and α -SMA

Double immunofluorescent staining was performed to determine the anatomic location of versican core protein (Figures 3G and 3J) and of the chondroitin-4-sulfate (C-4-S) chain (Figures 3H and 3K). In SAL-challenged lung, there was moderate staining for versican (Figure 3G) and C-4-S (Figure 3H) within the airway and vascular smooth muscle and in the alveolar wall. In OVA lung, we observed intense immunostaining for versican (Figure 3J) and C-4-S (Figure 3K) associated with airway and vascular smooth muscle. Close inspection indicated that dense staining also occurred in the alveolar and parenchymal regions. Staining for C-4-S was minimal in the epithelium, whereas the epithelium demonstrated immunoreactivity for versican. In general, the staining of C-4-S chains corresponded with the distribution of versican and most of the staining of C-4-S chains colocalized with versican, although areas of C-4-S staining not colocalizing with versican could not be ruled out. This implies that C-4-S chains may also be found in PGs other than versican, such as biglycan and decorin. Using a double immunostaining approach, we examined the cellular source and distribution of versican in SAL- and OVA-challenged lung. Compared with SAL-challenged lung (Figures 3M and 3N), OVA-challenged lung (Figures 3P and 3Q) demonstrated robust immunostaining for versican (Figure 3P) and α -SMA (Figure 3Q). The distribution of versican protein was associated with the distribution of α -SMA in the airway and blood vessel smooth muscle layers, suggesting a close relationship with smooth muscle cells.

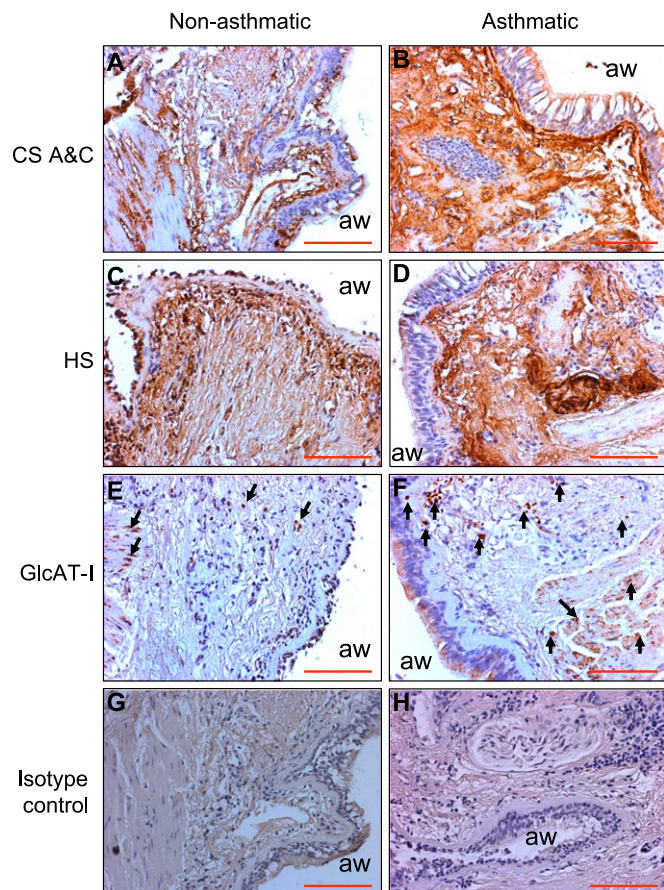


Figure 2. Immunohistochemical analysis of GAG chains in the airway wall of a subject without asthma (A, C, E, G) and a subject with severe asthma (B, D, F, H). (E and F) Immunostaining for CS types A and C (A, B), HS (C, D), and β 1,3-glucuronosyltransferase (GlcAT-I). The dark brown color is indicative of strong GAG expression and deposition. GlcAT-I expression was denoted by arrows. G and H are negative control from a subject without asthma and from a subject with severe asthma, respectively, stained with isotype-matched control antibody. Original magnification: $\times 200$. Scale bars = 100 μ m.

Versican immunoreactivity was also evident in areas outside smooth muscle layers, presumably corresponding to areas positive for fibroblasts or myofibroblasts.

OVA-Challenged Rat Lungs Contain Increased Amounts of Sulfated GAG Chains

Figure 4A shows the total content of GAGs in SAL and OVA lungs. The amounts of total sulfated GAG in OVA lungs was more than 2-fold the amount obtained from SAL lung. Figure 4A also demonstrates the composition of sulfated GAGs isolated from SAL and OVA lungs. The results show that 42% of the total GAGs in control lung were heparitinase resistant, most likely indicating CS/DS GAG chains. In contrast, HS accounted for 54% of the total sulfated GAGs in SAL lungs. The amount of CS/DS chains in OVA lung was 2.5-fold higher than in SAL lung, whereas the amount of HS was 2.1-fold more than that noted in SAL lung. Our findings also revealed that the relative proportion of CS/DS was increased in the OVA lung compared with SAL lung (47% in OVA versus 42% in SAL). Concomitant with the increase in CS/DS, there was a decrease in the relative percentage of HS in OVA lung (54% in SAL versus 49% in OVA).

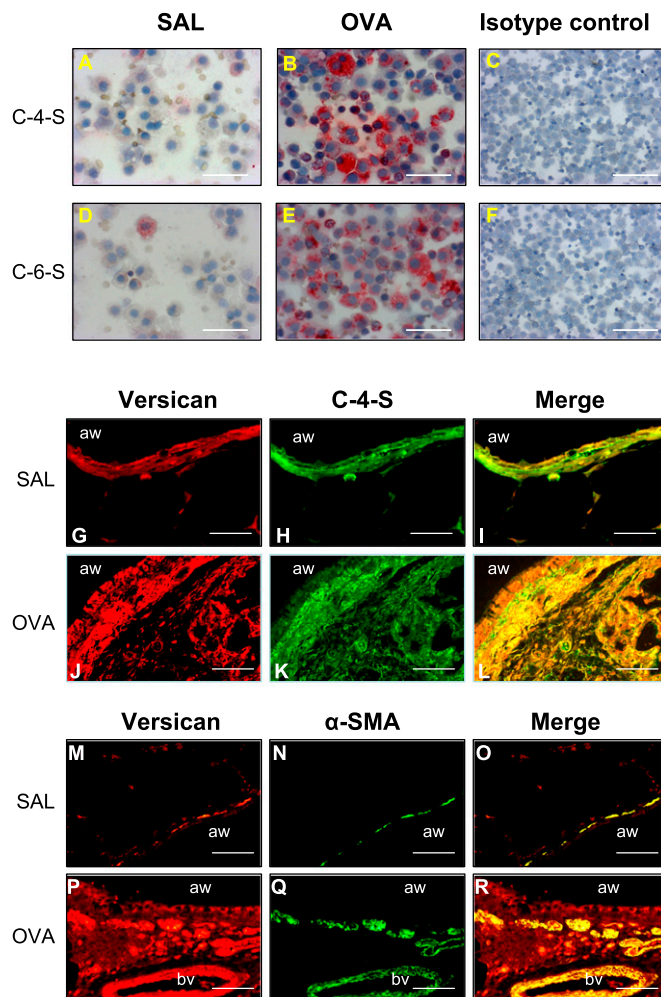


Figure 3. Immunostaining of GAGs in BAL cytospin preparations of SAL-challenged (A, D) and OVA-challenged (B, E) rats. Staining of cytospins for C-4-S (A, B) and C-6-S (D, E). C and F are negative controls from OVA-challenged rats stained with isotype-matched control antibody. Cells with bright red color were considered to have positive staining for GAG chains. Immunofluorescent colocalization of versican core protein and C-4-S in SAL-challenged (G, H, I) and OVA-challenged (J, K, L) rats. G and J show staining for versican (fluorescent red), H and K show staining for C-4-S (fluorescent green), and I and L show staining for the composite image. The bulk of C-4-S was found to be colocalized with versican. Photomicrographs of double immunofluorescent staining in SAL-challenged (M, N, O) and OVA-challenged (P, Q, R) rats for versican (M, P; fluorescent red) and α -smooth muscle actin (SMA) (N, Q; fluorescent green). Colocalization of versican and α -SMA is shown in the merged image (O, R; yellow). The majority of versican was found to be colocalized with α -SMA. In general, OVA-challenged rats displayed strong immunostaining for GAG chains and versican core protein showing increased PG-GAG deposition in their airways. Original magnification: $\times 200$. bv = blood vessel. Scale bars = 100 μ m.

Fluorophore-Assisted Carbohydrate Electrophoresis Analysis Displays Increased Content of HA and CS/DS Disaccharides in OVA-Challenged Rats

Figures 4B through 4E represent the fluorophore-assisted carbohydrate electrophoresis (FACE) profiles of GAG chains from the BALF of SAL- and OVA-challenged rats. Protein-free GAG chains digested with a mixture of hyaluronidase and chABC indicate the presence of internal unsaturated disaccharides from hyaluronic acid (Δ DiHA) and monosulfated

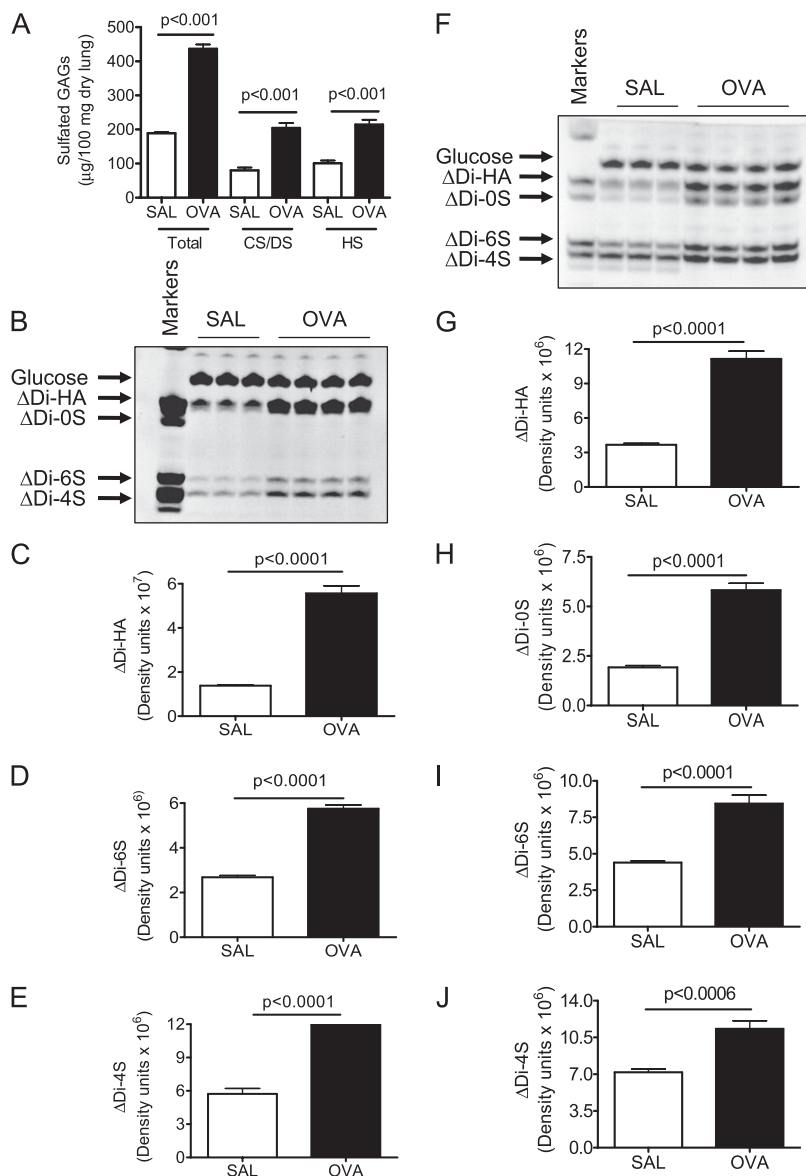


Figure 4. (A) Biochemical measurements of total sulfated GAG, chondroitin/dermatan sulfate (CS/DS), and HS content in SAL- and OVA-challenged rat lungs. Dried lung samples were used for enzymatic digestion with proteinase K, and aliquots of this digestion products were used for enzymatic analysis of the glycosaminoglycans with chondroitinase ABC or a mixture of heparinases to determine CS/DS or HS content as described in MATERIALS AND METHODS. Disaccharide composition (B, F) and densitometry quantitation (C–E, G–J) of hyaluronan (C, G), non-sulfated chondroitin (H), 6-sulfated chondroitin (D, I), and 4-sulfated chondroitin (E, J) disaccharides in bronchoalveolar lavage fluid (BALF) (B–E) and lung (F–J) of SAL- and OVA-challenged rats. Aliquots of BALF or lung tissues were digested with proteinase K, and their products were used for hyaluronidase and chondroitinase ABC digestion to produce HA and CS/DS disaccharides. Enzyme-liberated disaccharide products were fluorotagged and separated by fluorophore-assisted carbohydrate electrophoresis technique to identify and quantitate disaccharides. Disaccharides were recognized by coelectrophoresis with standards. Densitometry analysis of disaccharides was performed with image analyzer software (see MATERIALS AND METHODS). Values are mean $\pm$ SEM of six or seven animals and expressed in arbitrary densitometry units. Unpaired Student's *t* test was used for statistical analysis of the data. Δ DiOS = nonsulfated chondroitin-unsaturated disaccharide; Δ Di4S = chondroitin-4-sulfated unsaturated disaccharide; Δ Di6S = chondroitin-6-sulfated unsaturated disaccharide; Δ DiHA = HA-unsaturated disaccharide.

CS/DS chains (Δ Di4S and Δ Di6S). Although the FACE profiles in BALF from SAL- and OVA-challenged rats were similar, the levels of disaccharides derived from HA and CS/DS chains were significantly elevated in OVA-challenged rats. Densitometric analyses of FACE products revealed a nearly 4-fold increase in HA (Figure 4C) and 2-fold increases in 6-sulfated (Figure 4D) and 4-sulfated (Figure 4E) disaccharides in OVA-challenged rats compared with SAL-challenged control rats.

We then examined the disaccharide composition of HA and CS/DS chains in lung tissue. The electrophoretic pattern of OVA-challenged rats was the same as that of the SAL lung (Figure 4F). Results obtained after chABC digestion of CS chain revealed that the major unsaturated disaccharide is the mono-sulfated 4-disaccharide. In addition, 0- and 6-sulfated disaccharides were present in CS chain. Enzymatic digestion of GAG chains with hyaluronidase detected the presence of unsaturated Δ DiHA from hyaluronic acid. Compared with SAL-challenged rats, the data obtained from the analysis of HA and CS chains revealed relatively high levels of HA (Figure 4G) as well as 0-sulfated (Figure 4H), 6-sulfated (Figure 4I), and 4-sulfated (Figure 4J) chondroitin disaccharides in OVA-challenged rats. The

identity of the disaccharide bands was verified by coelectrophoresis with standard disaccharides.

Up-regulation of Glycosyltransferases and Sulfotransferases in OVA-Challenged Rats

Using quantitative real-time PCR (SYBR green technology), mRNA levels of glycosyltransferases and sulfotransferases were measured in lung tissues from SAL- and OVA-challenged rats (Figure 5). Expression of ribosomal protein S9 was measured as a control for RNA quality and content. Analysis of the expression of XT-I (Figure 5A), the enzyme responsible for initiation of GAG synthesis, demonstrated that XT-I mRNA levels were induced approximately 2-fold in OVA-challenged rat lung compared with the SAL-challenged group. Next, expression of GlcAT-I (Figure 5B), a rate-limiting enzyme in GAG synthesis, showed a similar increase in mRNA levels in OVA-challenged rats. These observations indicate that the induction of XT-I and GlcAT-I is equally affected during airway remodeling in OVA-challenged rats. Our findings also revealed that, in contrast to XT-I induction, mRNA levels of XT-II remained unchanged in OVA-challenged rats (data not shown).

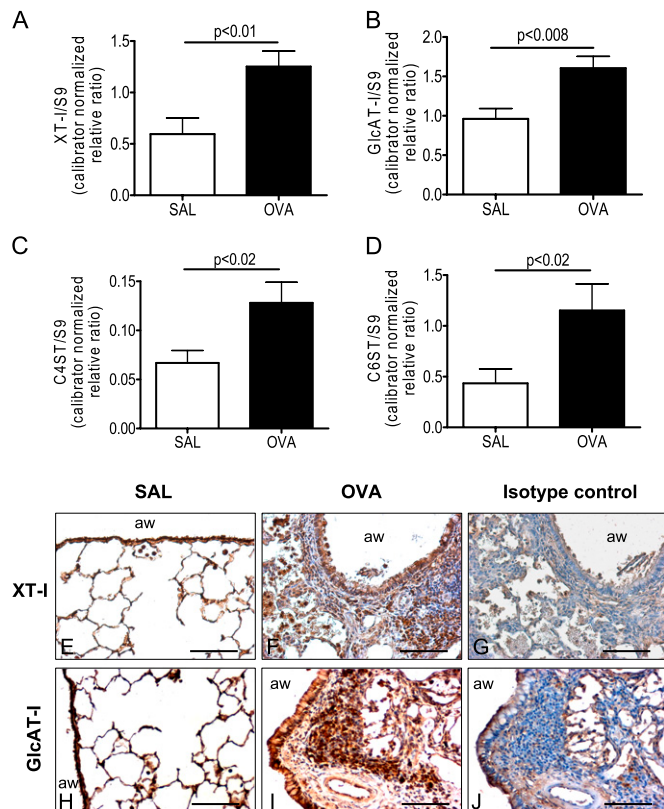


Figure 5. Expression levels of glycosyltransferases and sulfotransferases in SAL- and OVA-challenged rats. The lung tissues were analyzed for expression of XT-I (A), GlcAT-I (B), C-4-ST (C), and C-6-ST (D) mRNAs by quantitative RT-PCR ($n = \text{mean} \pm \text{SEM}$ of 6 or 7 observations). All values were normalized to ribosomal protein S9 mRNA and expressed as relative XT-I/S9, GlcAT-I/S9, C-4-ST/S9, or C-6-ST/S9 mRNA ratios. Unpaired Student's *t* test was used for statistical analysis of the data. (E–J) Photomicrographs of lung sections immunostained for XT-I (E–G) and GlcAT-I protein (H–J). OVA-challenged rats (F, I) showed a strong immunostaining for XT-I and GlcAT-I compared with SAL-challenged rats (E, H). The dark brown color is indicative of strong XT-I and GlcAT-I expression. G and J are negative control from OVA-challenged rats stained with isotype-matched control antibody. Original magnification: $\times 200$. XT-I = xylosyltransferase-I. Scale bars = 100 μm .

Next, we focused our attention on the expression of sulfotransferases, in particular C-4-ST and C-6-ST, which are enzymes that transfer a sulfate to the 4-*O*-position and 6-*O* position, respectively, of an N-acetylgalactosamine residue in CS chains. Our results showed that mRNA levels of C-4-ST (Figure 5C) and C-6-ST (Figure 5D) were significantly higher in OVA-challenged rats than in SAL rats. These findings are in good agreement with increased levels of 4- and 6-sulfated chondroitin disaccharides, as observed in IHC and FACE data.

OVA Challenge Results in Increased XT-I and GlcAT-I Protein Expression

We examined XT-I (Figures 5E–5G) and GlcAT-I (Figures 5H–5J) protein expression by IHC. The protein expression of XT-I and GlcAT-I paralleled the abundance of XT-I and GlcAT-I transcript in OVA- and SAL-challenged rats. Whereas weak immunostaining near nuclei in the cytoplasm of epithelial and inflammatory cells for XT-I and GlcAT-I was seen in SAL-challenged rats (Figures 5E and 5H), immunohistochemical analysis of OVA-challenged rats (Figures 5F and 5I)

demonstrated strong immunoreactivity for XT-I and GlcAT-I protein. Immunostaining was predominantly localized to airway structural cells as well as at sites of inflammation. Sections stained with isotype-matched control antibodies were negative (Figures 5G and 5J) for these proteins.

Increased Expression of PG Core Proteins in OVA-Challenged Rats

Next, we performed QRT-PCR analysis of PG core protein mRNA levels in SAL- and OVA-challenged rats. The mRNA of the large aggregating PG versican core protein was significantly higher (a 7.5-fold increase compared with SAL) in OVA-challenged rats (Figure 6A). OVA-challenged rats expressed 1.6-fold more decorin mRNA (Figure 6B) and 2.0-fold more biglycan mRNA (Figure 6C) compared with SAL-challenged rats. The mRNA data comparing OVA- versus SAL-challenged rats revealed a nearly 3-fold increase in lumican expression (Figure 6D). Western blotting experiments indicated that PG core protein expression (Figure E2) paralleled PG mRNA expression. In addition, perlecan expression was increased in OVA-challenged rats compared with SAL groups (Figure E3).

GAG Overproduction Is Relatively Steroid Insensitive

To assess the effect of budesonide on increased GAG production in OVA-challenged lung, tissue sections were immunostained for various GAGs. Analyses of lung sections revealed modest sensitivity of GAGs to budesonide treatment (Figure 7). With the exception of KS (Figure 7H), there was no change in the immunostaining of C-4-S (Figure 7B), chondroitin sulfate A and C (Figure 7D), and HS (Figure 7F) in OVA-challenged rats pretreated with budesonide.

DISCUSSION

In this study, we performed experiments to obtain a more comprehensive understanding of the alterations in ECM protein deposition, with a special focus on GAGs, during airway remodeling in OVA-challenged rats. According to our immunohistochemical analysis, CS and HS GAGs were abundantly expressed in the subepithelial compartment and in the basement membrane. The inflammatory cells adjacent to the basement membrane and in the alveolar interstitium also expressed HS. Finally, KS gave a distinct pattern of staining in the epithelial cells of the airways in SAL- and OVA-challenged rats. The KS immunoreactivity was strongest in the apical part of the epithelial cells, with no staining in the subepithelial and basement membrane region. Thus, most of the KS is probably produced in epithelial cells. Strong immunostaining of versican, together with its association in airway smooth muscle, indicate that these cells may contribute to the accumulation of versican in the airway wall during remodeling. Our results also showed substantial immunoreactivity for CS chains that was mostly localized to versican core protein. Together, this positive association supports our proposal that excessive production of GAG chains may be involved in the progression of remodeling via induction of matrix changes. The differential distributions of these various GAG chains indicate that they may serve different functions in the regions where they are abundantly expressed. Abnormal deposits of CS in and around the smooth muscle area may be related to stimulation of cell proliferation and migration in airway remodeling. Although increased production of HS may be important in the remodeling of basement membrane in the airways, increased expression of epithelial KS may be related to

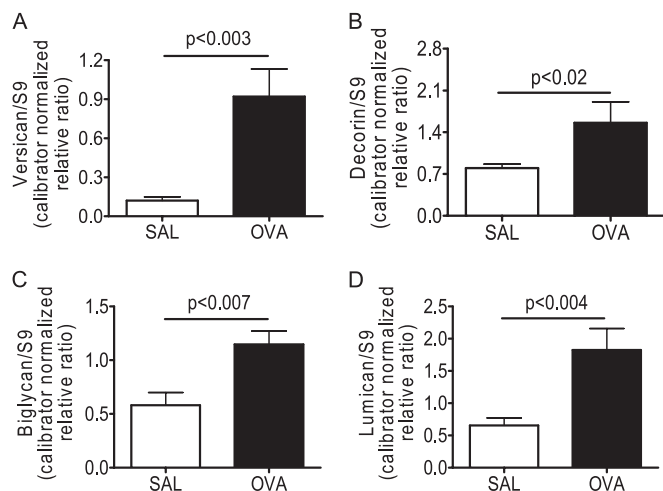


Figure 6. Expression levels of various extracellular matrix proteins in SAL- and OVA-challenged rats. The lung tissues were analyzed for expression of versican (A), decorin (B), biglycan (C), and lumican (D) mRNAs by quantitative RT-PCR ($n = \text{mean} \pm \text{SEM}$ of 6 or 7 observations). All values were normalized to ribosomal protein S9 mRNA and expressed as relative versican/S9, decorin/S9, biglycan/S9, or lumican/S9 mRNA ratios. Unpaired Student's *t* test was used for statistical analysis of the data.

increased airway secretions. It is also interesting to observe that the expression of C-4-S and C-6-S chains was higher in BAL cytospin preparations of OVA-challenged rats compared with SAL-challenged rats. Although the function of these CS chains in these inflammatory cells is unknown, their increased expression might be related to regulation of growth factor and cytokine activities (3, 9, 31–36) during inflammation and tissue repair. To determine the clinical significance of our animal data, we immunostained human biopsy samples for GAGs. In accord with our previously reported findings on PG core proteins (21), asthmatic airways demonstrated increased expression of CS, HS GAGs, and GlcAT-I protein compared with those from a subject without asthma. These findings strengthen our hypothesis that anabolic changes leading to increased deposition of GAGs may contribute to airway remodeling in asthma.

Our immunohistochemistry findings were further supported by the data on the biochemical quantification of GAGs. Biochemical measurements of total sulfated GAG, as well as the quantities of CS/DS and HS GAG, were higher in OVA-challenged lungs than in SAL-challenged lungs. Moreover, lungs from OVA-challenged rats showed a relative increase in the percentage of CS/DS GAGs compared with the levels found in SAL-challenged rats. This finding was paralleled by a decrease in percentage of HS GAGs, indicating the occurrence of qualitative and quantitative abnormalities of GAG chains in OVA-challenged rats. These observations have not been reported by other investigators and thus demonstrate a particularly interesting part of the airway remodeling process. The mechanism that determines this compositional difference is unclear. However, changes in the GAG profile may have significant functional effects resulting not only from a decrease in the relative proportion of HS GAG chains but also from the deposition of additional CS/DS GAGs. The presence of increased CS/DS GAGs may impart an increased negative charge that could interfere with the organization and proper hydration of the ECM (3, 37) of the airway wall in asthma. Versican, the large aggregating PG in the lung (1, 4), is a carrier of CS chains, and the expression of this core protein has been shown to be up-regulated in human asthma (20). We also found increased deposition and colocalization

of CS chains to versican core protein in OVA-challenged rats. These findings indicate that airway remodeling in OVA-challenged BN rats may result in “different” PG-GAG composition, which may favor matrix and architectural remodeling and disease progression.

In the next part of our investigation, we examined details of the sulfation levels in the CS/DS GAG chain. In doing so, we identified structural differences of CS/DS chains between SAL- and OVA-challenged rats. The disaccharides obtained from OVA-challenged lung revealed significant increases in 4-, 6-, and 0-sulfated chondroitin disaccharides compared with that derived from SAL-challenged lungs. Furthermore, the disaccharide level of HA was increased in OVA-challenged lungs. An increase in the amount of HA, coupled with high concentrations of CS chains and versican, results in large versican–HA aggregates within the thickened airway wall, leading to remodeling of the structure of the airways. These changes in structure likely lead to functional defects.

The GAG disaccharide composition presented here shows some interesting parallels and differences between airway remodeling in asthma and pulmonary fibrosis. For example, in lung fibrosis, we have recently shown (29) that 4- and 6-sulfated chondroitin disaccharides were increased without any significant change in 0-sulfated chondroitin disaccharide. However, the data presented here indicate that during airway remodeling an increase in CS disaccharides is not limited to 4- and 6-sulfated disaccharides but also includes 0-sulfated chondroitin disaccharide. This implies that sulfation of GAG chains may be differentially controlled in these two remodeling diseases. The biological function of GAG chains or the importance of GAG sulfation is less well understood in asthma. However, a previous study from our laboratory demonstrated that the ability of decorin to reduce airway smooth muscle proliferation was compromised when decorin-coated matrices were digested with chondroitinase ABC, an enzyme that cleaves CS/DS GAG side chains, indicating that CS/DS chains may be crucial to the anti-proliferative and proapoptotic properties of decorin (38). Furthermore, altered sulfation of GAG chains could influence interactions with ECM proteins, growth factors, enzymes, and enzyme inhibitors as well as cell adhesion (31, 39–42), which may have a direct or indirect impact on ECM remodeling in asthmatic airways.

It is unclear what mechanism underlies altered GAG expression and sulfation in OVA-challenged rats. Therefore, we investigated the hypothesis that increased expression and alterations in GAG sulfation during airway remodeling are biosynthetically generated. We studied the XT-I and GlcAT-I expression as model enzymes for the analysis of GAG expression. Both are important enzymes; experiments in which XT-I and GlcAT-I levels are genetically modified have shown that these GTs have a strong regulatory role in GAG synthesis and deposition (43–47). In support of our hypothesis, gene expression studies confirmed that the levels of the XT-I and GlcAT-I transcript were higher in OVA lung, and the increased accumulation of GAGs, which inevitably would follow the induction of the two biosynthetic enzymes, were be equally complemented by up-regulation of the C-4-ST and C-6-ST genes. This would result in increased GAG concentrations with increased sulfation of 4- and 6-sulfated chondroitin disaccharides, which is consistent with our IHC and FACE analyses showing increased expression of 4- and 6-sulfated CS chains and CS disaccharides. Subsequently, lung sections stained for XT-I and GlcAT-I enzymes demonstrated intense expression of these proteins in OVA-challenged rats. We were unable to study the expression of C-4-ST and C-6-ST at the protein level due to a lack of commercially available antibodies. Our findings that GT and ST mRNA overexpression coordinate

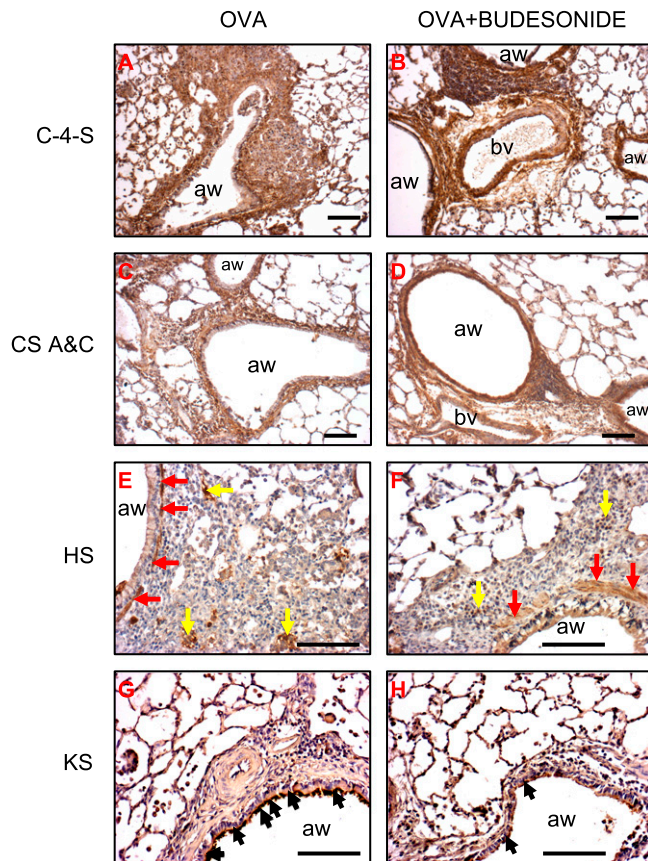


Figure 7. Effects of budesonide on OVA-induced GAG deposition. Immunohistochemical analysis of GAG chains in the airway wall of OVA-challenged (A, C, E, G) or OVA-challenged + budesonide-treated (B, D, F, H) rats. Immunostaining of C-4-S (A, B), CS A and C (C, D), HS (E, F), and KS (G, H). Immunostaining for all GAG chains was increased in the airways in OVA-challenged rats. Immunostaining was noted in the sub-epithelium, basement membrane, and airway smooth muscle region for C-4-S, CS A and C, and HS. Immunoreactivity for KS was observed apically in the surface epithelium (black arrows). Yellow arrows depict immunostaining for HS in areas of inflammation. Red arrows show HS expression in the peribronchial area. The dark brown color is indicative of strong GAG expression and deposition. Budesonide treatment did not result in inhibition of OVA-induced increases in C-4-S, CS A and C, and HS deposition but reduced KS expression. Original magnification: $\times 200$. Scale bars = 100 μm .

with subsequent changes in expression and sulfation of GAGs have not been reported previously in experimental or clinical asthma studies.

Remodeled airways in OVA-challenged rats also exhibited increased expression of PG core protein-specific genes. In the OVA-challenged rats analyzed, mRNA and protein levels of large (versican) and small (biglycan, decorin, and lumican) PGs showed a striking correspondence with the alterations in CS/DS and KS chains. Earlier studies have demonstrated a significant increase in the expression of these PGs at the protein level in asthmatic lungs and animal models (20–24). Taken together, these data show that the PG-GAG matrix of the airway wall in allergen-challenged models is being synthesized at a considerably higher rate than that in normal controls. Our data also suggest that airway remodeling in OVA-challenged rats results in the up-regulation of all pertinent components (GAG chains, GAG biosynthetic enzymes, and core proteins) involved in PG-GAG formation.

PG-GAGs are important in airway and lung ECM homeostasis and would be expected to influence the mechanical function of the airways in allergen-challenged BN rats. Although we did not examine this directly in the current experiment, studies performed previously in this laboratory in the same model (i.e., chronically allergen-challenged, sensitized BN rats) have shown that lung physiologic changes occur coincident with the currently reported alterations in PG-GAGs (27). The pathophysiological and structural changes that occur in this model include airway hyperresponsiveness to inhaled methacholine, increased airway smooth muscle growth, increased total airway wall area, goblet cell hyperplasia, and airway inflammation (27, 48). Large aggregating proteoglycans, including versican, contain numerous hydrophilic GAG chains that draw ions and water into the matrix and thereby influence tissue turgor (1). In addition, hyaluronan forms a hydrated matrix because of its polyanionic core structure, and formation of large hyaluronan-versican aggregates could also contribute to the local compressive modulus of the lung. Data from our laboratory suggest that alterations in PG-GAGs are associated with susceptibility to changes in airway mechanics and the viscoelastic properties of the lung tissues (49, 50). Our findings are consistent with other studies of PG expression in the airways of humans. Huang and colleagues (20) reported that PG deposition was significantly increased in the airways of subjects with asthma as compared with control subjects and showed a significant correlation between the amount of PG in the airway wall and airway responsiveness. All of these studies indicate that alterations of PG-GAGs in the airway wall likely affect airway mechanical properties and airway responsiveness (51).

Corticosteroids are often used to treat asthma because of their ability to reduce inflammation and airway hyperresponsiveness (52). Because increased deposition of ECM proteins is a feature of airway remodeling in asthma, several studies have investigated the efficacy of corticosteroids to inhibit ECM overproduction in asthmatic airways. *In vivo* studies conducted in the BN rat model have characterized fluticasone as an inhibitor of fibronectin deposition after prolonged repeated allergen challenge (53). A recent study by Siddiqui and colleagues (27) from our laboratory reported that budesonide treatment inhibited airway smooth muscle growth and central airway goblet cell hyperplasia after OVA challenge in the BN rat. In that study, budesonide also inhibited small but not large airway total wall area. We examined whether budesonide treatment had any effect on overproduction of GAGs in OVA-challenged rats. Generally, most GAG subtypes appeared to be refractory to budesonide treatment in the OVA-challenged rats. Budesonide did not inhibit excess C-4-S and CS types A and C deposition in the OVA-challenged rat airway. HS appeared to be reduced in areas of inflammation but not in the peribronchial area. However, KS deposition appeared to be decreased. The reduction by budesonide of KS expression is interesting to consider in light of the finding that budesonide suppressed goblet cell hyperplasia in the OVA-challenged BN rat (27). Given that increases in goblet cells and excess mucus production characterize asthma and that KS has been shown to be associated with airway mucus (54), it is possible that inhibition of goblet cell number and mucus secretion by budesonide could account for the decrease in KS in steroid-treated, OVA-challenged rats. These data provide evidence for a differential susceptibility of GAGs to budesonide treatment. Collectively, the effect of steroid on GAG deposition was mild, which is in agreement with what has been described in animal models and human asthma (55–58).

In summary, we addressed the question of whether airway remodeling was associated with altered expression of GAGs

and their biosynthetic apparatus and demonstrated increased deposition and sulfation of GAGs in OVA-challenged rats, which was linked with increased mRNA levels of glycosyltransferases and sulfotransferases. Further investigation of the anabolic changes is likely to provide an insight into the role played by glycosyltransferases and sulfotransferases in promoting PG-GAG deposition, an event that appears to be central in driving structural changes and altered airway function in asthma.

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