ERJ Express. Published on October 18, 2006 as doi: 10.1183/09031936.00047905

DIFFERENCES IN PROTEOGLYCAN DEPOSITION IN THE AIRWAY OF MODERATE AND SEVERE ASTHMATICS

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Running title: differences in PG deposition

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ABSTRACT

Excess deposition of proteoglycans (PG) has been described in the sub-epithelial layer of the asthmatic airway wall. However, less is known about deposition in the airway smooth muscle (ASM) layer, and whether the pattern of deposition is altered depending upon disease severity.

Endobronchial biopsies were performed in patients with severe or moderate asthma (defined using ATS criteria) and in control subjects. Biopsies were immunostained for the PGs, biglycan, lumican, versican and decorin. PG deposition was measured in the subepithelial and ASM layers, the former by calculating the area of positive staining, and the latter, by determining the % area using point counting.

Immunostaining for PG was prominent in biopsies from both moderate and severe asthmatics, as compared to control subjects. While the amount of PG in the sub-epithelial layer was no different in the two asthmatic groups, the % area of biglycan and lumican in the ASM layer was significantly greater in moderate vs severe asthmatics ($p < 0.01$ and 0.05, resp.).

Differences in the deposition of PG within the ASM layer of moderate vs severe asthmatics could potentially impact on the functional behaviour of the airway smooth muscle in these two groups of patients.

Key words: airway smooth muscle, biglycan, decorin, lumican, versican,

INTRODUCTION

Remodeling of the airway wall is well described in asthmatic patients (1). Studies have shown thickening of the reticular basement membrane and increased matrix deposition in the sub-epithelial layer of the conducting airways (2-5). Collagen and glycoproteins, such as fibronectin, account for some of these changes; proteoglycans also contribute to the remodeling (6). The precise link between remodeling and asthma severity has not, as yet, been established. Benayoun and colleagues (7) noted an increase in collagen type III deposition in the bronchial submucosa of patients with severe, persistent asthma. Studies from this laboratory (5) showed a positive relationship between airway wall thickening and asthma severity. On the other hand, Chu and colleagues (3) were unable to document differences in collagen III immunostaining in airways from patients with asthma of varying severity. Further, Niimi and colleagues (8) showed that airway wall thickness, as assessed by computerized tomography, and airway reactivity were inversely correlated. Whether other extracellular matrix (ECM) molecules, such as proteoglycans, are important in contributing to asthma severity has not been investigated. Further, whether matrix deposition is altered in the smooth muscle layer in moderate vs severe asthmatics has not been addressed.

Proteoglycans are macromolecules consisting of a protein core and glycosaminoglycan (GAG) side-chains. Different sub-classes of PGs have been described including hyalectins, such as versican, which are large molecules with many GAG side-chains, and small leucine rich PG (SLRP), eg., decorin, biglycan, and lumican. Proteoglycans subserve a number of important biologic functions (9). Versican, because of the high ionic charge of its multiple GAG side-chains, plays a critical role in determining the water content of extracellular matrices, and influences tissue viscoelastic behavior. Decorin and biglycan are molecules which bind to collagen and affect collagen fibrillogenesis and matrix assembly. These molecules also bind different growth factors, such as transforming growth factor

(TGF) β and fibroblast growth factor, and modulate their ability to influence cell proliferation and matrix deposition (10).

Roberts (11) made the observation that, in patients dying of asthma, the airway wall showed prominent staining for biglycan, decorin and versican. We have shown, in endobronchial biopsy specimens obtained from mild asthmatics, that deposition of the PGs, versican, biglycan and lumican was increased in the sub-epithelial layer of the airway wall, as compared to biopsies obtained from normal volunteers (6). Reddington and co-workers (12) showed co-localization of decorin and TGFβ_1, in the sub-epithelial layer of the airway wall; however, the overall pattern of deposition was no different in biopsy specimens from asthmatic and control subjects. A recent report in mild asthmatic subjects (13) showed a decrease in decorin in the lamina propria as compared to normal controls; conversely, biglycan was increased. Hence, the nature of PG deposition in the asthmatic airway wall is far from clear.

The increased PG expression in the asthmatic airway wall could have important functional consequences. Thickening of the airway wall may lead to increased airway resistance because of decreases in luminal diameter, and to increased airway responsiveness because of the effects of a thickened airway wall on airway smooth muscle shortening and subsequent airway narrowing (14). In a study of mild atopic asthmatics, PG deposition in the sub-epithelial layer was positively correlated with airway responsiveness (6). On the other hand, increased matrix deposition in the smooth muscle layer itself, could theoretically result in decreased smooth muscle constriction (15;16). A recent review by McParland et al (15) promotes the hypothesis that remodeling could have modulating effects on airway narrowing; stiffening of the airway wall could lead to decreased compressibility, while increased matrix within the smooth muscle layer could increase the impedance to airway smooth muscle shortening. Much would depend on the precise mechanical characteristics of the deposited

matrix. Hence, the site of enhanced matrix deposition and the specific mechanical properties of the matrix itself, may be critical determinants of the impact of remodeling on airway physiology.

In order to examine changes in proteoglycans, and their contribution to airway wall remodeling in moderate and severe asthmatic patients, we used endobronchial biopsy to obtain specimens of the airway wall, and immunohiostochemistry to characterize proteoglycan deposition in the different layers of the airway wall sampled.

METHODS

Participants

Twenty-seven asthmatic patients ranging in age from 22 to 66 years and six control subjects ranging in age from 20 to 63 years participated in this study (Table 1). Fourteen severe and thirteen moderate asthmatics of both sexes were recruited from the Difficult Asthma Study, conducted at the Montreal Chest Institute, McGill University, and at the Hôpital du Sacre-Coeur, Université de Montréal. All patients had standard pulmonary function testing performed.

Asthma was diagnosed by a respirologist and was confirmed by an improvement in FEV_1 (forced expiratory volume in one second) of 12% or greater, with an absolute increase of at least 200 ml from baseline, after administration of a β-adrenergic agonist; or by an improvement of FEV_1 of 20% or greater after a steroid trial.

Severe asthma was defined according to criteria adapted from an American Thoracic Society workshop on refractory asthma (17). Severe asthmatic patients had either treatment with daily oral steroids for more than 50% of the previous 12 months, or treatment with high dose inhaled steroid (more then 1000 μg fluticasone or equivalent per day) and at least one other drug (long-acting β-agonist, leukotriene receptor antagonist, or theophylline)

continuously over the previous 12 months. In addition, they had to meet at least 2 of the following criteria: daily use of short-acting B-agonist, persistent airflow obstruction as documented with pre-bronchodilator FEV_1 less than 70% and FEV_1/ FVC ratio less than 80% predicted, at least one urgent care visit in the previous 12 months, three or more courses of oral steroids in the previous 12 months, prompt deterioration with <25% dose reduction of steroids, or near fatal asthma event within the last 3 years.

Moderate asthmatics met all of the following criteria: well-controlled asthma with 200-1000 μg/day of fluticasone or equivalent, with or without the use of a long-acting β-agonist, 2 or less oral steroid courses in the previous 12 months but none in the past 3 months and with the total number of days on oral steroids not to exceed 30 days in the previous 12 months, FEV_1 more than 70% predicted and more than 90% of personal best from the previous 2 years, no more than one non-scheduled urgent care or office visit in the previous 12 months.

Individuals were excluded if they had other known pulmonary disease or presence of other major co-morbid disease that might affect asthma disease activity, such as HIV, metastatic cancer or congestive heart failure. Informed consent was obtained before bronchoscopy was performed. The study was approved by the ethic committees of the McGill University Health Centre and the Research Centre of Hôpital du Sacre-Coeur of Université de Montréal.

Procedures

Bronchoscopy was performed according to American Thoracic Society guidelines. Four to six biopsy specimens per patient were taken at the carina of segmental bronchi using cup forceps. Biopsy specimens were placed in TBS solution and immediately transported to the laboratory for processing. For biglycan, lumican and decorin immunostaining, tissues

were fixed in 4% paraformaldehyde and embedded in paraffin. Tissues were then sectioned (5-μm-thickness) and placed on glass slides coated with poly-L-lysine (0.1%) and baked overnight at 37^{O}C. For versican immunostaining, tissues were snap frozen in isopentane, blocked in optimum-cutting-temperature embedding medium, sectioned (5-μm-thickness) and then fixed in acetone/methanol. Some of the biopsy specimens thus obtained were used in another study (18). Only asthmatic subjects had tissue stained for versican, as frozen biopsy specimens were not available in control subjects.

Immunohistochemistry

Slides were stained with primary antibodies to biglycan, (polyclonal rabbit, anti-human, 1:500), lumican (polyclonal rabbit, anti-human, 1:500, both generous gifts of P. Roughley, Shriners Hospital, McGill University), decorin (polyclonal rabbit, anti- mouse, 1:1000, Hybridoma Bank) or versican (monoclonal mouse, anti-human, 1:500, Hybridoma Bank). Secondary antibodies used were swine antibody to rabbit immunoglobulin (1:100, DAKO) or rabbit antibody to mouse immunogloblulin (1:60, DAKO) (versican). Steptavidin-alkaline phosphatase (1:200, DAKO) or mouse alkaline phosphatase/anti-alkaline phosphatase (1:60 dilution, DAKO) (versican) was used for detection. All immunostaining was developed with Fast Red (Sigma, Toronto, Ontario). Slides were counterstained with Gill II haematoxylin and coverslips applied. Positive staining appeared red under bright-field illumination. For negative controls, sections were processed in the absence of primary antibody. Not all biopsy samples were stained for all proteoglycans. The number of biopsy specimens examined for each PG ranged from four to six. Some patients had separate biopsy samples stained for more than one PG.

Morphometry

The slides were examined using an Olympus light microscope (Carson Group, Markam, Ontario) and images captured with commercial software (Image-Pro Plus, Silver Spring, MD). To detect positive staining in the subepithelial layer, 200 x magnification was used. The subepithelial layer was defined as including the reticular basement membrane and extending down to the smooth muscle layer. Blood vessels and mucous glands were excluded from the analysis. All the subepithelial area under identifiable basement membrane in the biopsy specimen, was included in the analysis. Positive staining was established by determining a colour threshold, and was quantified either by tracing the area using a digital pen (Wacom, Vancouver, USA) or using image analysis software (control subjects). Area (in μm^2) was standardized for the basement membrane length squared (bm^2). (Basement membrane length was squared so that an area was corrected for an area.) Values were calculated using SigmaScan (Jandel Scientific, Corte Madera, CA). To detect positive staining in the smooth muscle layer, an eyepiece graticule was applied. (At 100x magnification, the area of the graticule was 1.0 mm^2). The amount of PG within or overlying the smooth muscle layer was expressed as the percentage of positive staining overlapping the cross-points of a 121 point grid. Measurements were made at a magnification of 1000x in ten randomly chosen fields, per biopsy. We included those areas where ASM was clearly identified, and excluded adjacent connective tissue.

We used different morphometric techniques to measure PG in the sub-epithelial and smooth muscle layers for the following reasons. When there is a well-defined area of positive staining, such as was the case in the sub-epithelial layer, one can measure the area and standardize to basement membrane. When the area of positive staining is more diffuse, such as was the case in the ASM layer, it is more difficult to measure total area. As the reference volume was well defined, we used an area ratio estimate, ie, point counting. Of note, we did

not directly compare PG results between the sub-epithelial and smooth muscle layers. Rather, we used the same technique to compare the signal between moderate and severe asthmatics.

Statistical analysis

Comparisons of PG deposition among severe and moderate patients, and normal controls were performed using analysis of variance (ANOVA) and Bonferroni's multiple comparison test. Where data were not normally distributed, a Kruskal-Wallis test followed by Dunn's multiple comparison test were applied. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Characteristics of the twenty-seven asthmatic patients recruited for study: fourteen with severe asthma, and thirteen with moderate asthma, and the six control subjects are shown in Table I. The average FEV_1 (% predicted), in the severe group was 63.6 ± 6.8, in the moderate group was 87.5 ± 4.7% and in the control group was 109.8 ± 7.1%.

Immunohistochemistry

Positive staining for biglycan, lumican, versican and decorin was identified in all airway specimens of both severe and moderate asthmatic patients. Positive staining for biglycan, lumican and versican was present in both the subepithelial and the smooth muscle layers; however, the pattern of staining differed between the two populations (Figure 1A-C). Whereas staining for biglycan, lumican and versican was prominent in the smooth muscle layer in moderate asthmatics (Figure 1B); staining for PG within the smooth muscle layer was relatively sparse in severe asthmatics (Figure 1A,C), although still greater than that observed in control subjects (Figure 1D). Staining for these PGs within the sub-epithelial layer was marked in both moderate and severe asthmatic patients. While decorin staining was

evident in the reticular basement membrane, it was minimal within the smooth muscle layer. Biopsy specimens from both moderate and severe asthmatics showed substantial amounts of smooth muscle. Only a small proportion of biopsies had negligible ASM (~11%).

Morphometry

Quantification of the area of positive staining in the subepithelial layer was normalized by basement membrane length squared (A/bm^2). Measurements for biglycan, lumican, versican and decorin are shown in Figure 2. Staining for lumican was significantly increased in severe asthmatics vs normal controls ($p < 0.05$) and there was a strong trend for differences among the three groups for biglycan ($p = 0.06$) (Kruskal-Wallis). There were no significant differences in immunostaining for decorin or versican among the moderate and severe asthmatic, and control groups.

Proteoglycan expression in the smooth muscle layer was measured by point counting and is shown in Figure 3. In the smooth muscle layer of moderate asthmatic patients, there was significantly greater protein expression of biglycan and lumican as compared to severe asthmatics ($p< 0.01$ and 0.05, respectively) and normal controls ($p < 0.001$ for both PG) (ANOVA). Minimal staining for decorin was found in the smooth muscle layer in airways from all three groups.

DISCUSSION

The results of this study show that remodeling of the airway wall is different in moderate vs severe asthmatics; while PG deposition in the subepithelial layer was similar in the two groups of asthmatic patients, moderate asthmatics had significantly greater proteoglycan deposition within the smooth muscle layer as compared to severe asthmatics, and normal controls. Such a distribution of matrix in the airway wall could potentially modulate airway narrowing in the moderate asthmatic group, by impeding airway smooth

muscle shortening. These data are consistent with the recently advanced hypothesis that remodeling of the airway wall in asthma could serve a protective role (15).

Enhanced deposition of matrix within the sub-epithelial layer of the asthmatic airway wall is well established (1;2). Studies in asthmatics of all severities, have shown prominent thickening of the reticular basement membrane, and sub-epithelial fibrosis (1-3). We have shown in mild asthmatics, that deposition of the PGs, versican, biglycan and lumican, was increased in the subepithelial layer of the airway wall, as compared to that of normal subjects (6). De Kluijver and colleagues (13) have recently reported that, while biglycan was increased in the lamina propria of airways from mild asthmatic patients as compared to controls, decorin was decreased. Roberts (11) examined autopsy specimens of patients who died of asthma, and showed that the airway wall stained prominently for versican, biglycan and decorin; staining was particularly evident in the sub-mucosa and around smooth muscle cells. De Medeiros and colleagues (19) recently showed that in patients dying of fatal asthma, versican content in the internal area of both large and small airways was increased as compared to controls. Some information is also available from *in vitro* studies. Fibroblasts obtained from endoscopic biopsies of airways of mild asthmatic patients, demonstrated increased production of versican, perlecan and biglycan, as compared to cells isolated from normal controls (20). We have shown that message for decorin is upregulated in these same cells (21). The data of the current experiment corroborates these findings; the expression of PG was increased in the sub-epithelial layer in patients with asthma as compared to control. Staining for the proteoglycans, biglycan and lumican within the smooth muscle layer was significantly increased in patients with disease of moderate severity as compared to control.

The potential correlation between changes in airway structure and airway function in asthma has been addressed previously. The link between increased collagen deposition in the sub-epithelium, and asthma severity has been examined; the results have been conflicting.

Benayoun and colleagues (7) described an increase in collagen III immunostaining in the sub-epithelial layer of the airways only in asthmatics with severe disease; however, Chu and colleagues (3) were unable to document an association between sub-mucosal collagen deposition and degree of disease. Previous work from our laboratory in which moderate and severe asthmatics were grouped together, showed relatively greater amounts of sub-mucosal collagen deposition in these patients, as compared to subjects with mild disease and normal controls (5). This observation is further addressed by the results published recently in a group of patients overlapping those of the current study (18). While the percentage of subepithelial fibrosis was no different between moderate and severe asthmatics, the distance between the ASM and the epithelium, was significantly decreased in severe asthmatic patients. Further, airway smooth muscle mass was also greater in severe patients. However, changes in the extracellular matrix at the level of the smooth muscle and the relation to disease severity have not been previously examined.

In previous work, we have shown a significant correlation between proteoglycan deposition in the sub-epithelial layer of the airway wall and clinical disease. A significant correlation was determined between sub-epithelial deposition of PG and airways responsiveness in mild, atopic asthmatics. Similarly, Westergren-Thorrson et al (20) showed in fibroblasts isolated from endoscopic biopsies, that cells from asthmatic patients with the greatest degree of hyperresponsiveness, produced larger amounts of proteoglycans.

Our current finding that patients with more moderate asthma, have greater PG deposition within the smooth muscle layer, as compared to severe asthmatics, is perhaps surprising. Further, there is little information available in the literature on PG in ASM in control subjects. The data of the current experiment demonstrate minimal deposition of PG within the ASM layer under non-disease conditions. The potential effects of increased PG within the ASM layer can be best understood by considering theoretical models (14;22;23).

Generally, the accepted idea has been that increased matrix deposition in the airway wall would lead to increased airway constriction; a thickened sub-mucosal layer would result in a greater degree of luminal narrowing for a given degree of airway smooth muscle constriction. This hypothesis has been more recently countered by the argument that increased matrix deposition within the smooth muscle layer could have a modulating effect (15;16;24). Excessive matrix could increase the impedence to airway smooth muscle constriction, and thereby decrease the actual length change in the smooth muscle for a given degree of contractile stimulation. Along these lines, Bramley and colleagues (25) demonstrated that excised tracheal strips exposed to proteolytic digestion of the matrix, showed enhanced shortening in response to contractile stimulation. A recent study by Niimi and colleagues (8) showed that airway wall thickening, measured by computer tomography, correlated negatively with airway reactivity in patients with asthma. Our data, showing greater matrix deposition within the smooth muscle layer, in asthmatics in whom clinical disease was less severe, are the first direct evidence in patients to support the hypothesis that matrix deposition within the ASM layer might modulate disease severity. A further piece of evidence can be drawn from the recent autopsy study in fatal asthmatics published by de Medeiros et al (19). While these authors did not specifically quantify proteoglycans within the airway smooth muscle layer, airway photomicrographs from fatal asthmatic patients showed a conspicuous absence of PG immunostaining in the ASM layer.

Unfortunately, in the current study, we were not able to measure airway responsiveness in the two groups of patients, because of the severity of their underlying disease, and the degree of baseline airway narrowing. It would have been very interesting to correlate responsiveness with the amount of PG deposition within the ASM layer.

One potential confounding factor relates to the amount of steroid therapy received by these two groups of patients. While steroids could potentially affect PG deposition (26), there

is no *a priori* reason to expect that steroids would differentially affect deposition in the subepithelial vs smooth muscle layer.

Proteoglycans are likely candidates to affect the mechanical impedence of the airway smooth muscle layer. Proteoglycans consist of a protein core to which are attached GAG sidechains. These hydrophilic sidechains affect tissue turgor by altering the water content of the tissues and thereby, the mechanical behaviour of the system (9). In addition, proteoglycans, modulate collagen fibrillogenesis, and may affect tissue mechanics via effects on collagen fibril formation (9). We have conducted experiments in both *in vivo* and *in vitro* models which demonstrate that alterations in proteoglycans affect tissue viscoelastic behaviour. Experiments in isolated parenchymal strips showed that specific degradation of PG altered tissue viscoelastic behaviour (27). Both tissue elastance and resistance were decreased after digestion of GAG sidechains. In *in vivo* experiments on mice deficient in the proteoglycan, decorin, the effects of lack of decorin on the mechanical characteristics of both the parenchymal tissues and airway mechanics were examined. Lung compliance was increased and airway resistance decreased in these decorin deficient mice (28). Hence, changes in proteoglycans in the asthmatic airway wall would likely result in alterations in the mechanical behaviour of the airway. Whether that alteration is in a positive or negative direction, may depend on the specific layer of the airway wall in which deposition is enhanced.

A further question is the effect of PG on the functional or phenotypic characteristics of the adjacent structural cells. Again, some information is available from *in vitro* studies. Hirst et al (29) have shown that various ECM proteins, such as collagen and fibronectin, have the capacity to affect ASM proliferation. Johnson and colleagues (30) have reported that ECM proteins secreted by asthmatic ASM, which likely include PG, enhance ASM

proliferation, in an autocrine fashion. The precise effects of PG on ASM proliferation and/or contractility remain to be determined.

In conclusion, we have shown that moderate asthmatic patients have relatively greater deposition of proteoglycan in the smooth muscle layer of the airway wall, than do patients with more severe disease. This enhanced deposition of matrix could potentially serve a modulating role, protecting against excessive smooth muscle constriction. It could also impact on the phenotypic behaviour of the smooth muscle itself. These are some of the first data collected from patients which support the hypothesis that airway remodelling in asthma, may be a "friend, not a foe" (15).

ACKNOWLEDGEMENTS

Supported by the Edith Strauss Foundation, Canadian Institutes of Health Research, the JT Costello Memorial Research Fund and Glaxo Smith Kline.

Reference List

(1) Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. Am J Respir Crit Care Med 2001 Nov 15;164(10 Pt 2):S28-S38.

(2) Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. Lancet 1989 Mar 11;1(8637):520-4.

(3) Chu HW, Halliday JL, Martin RJ, Leung DY, Szefler SJ, Wenzel SE. Collagen deposition in large airways may not differentiate severe asthma from milder forms of the disease. Am J Respir Crit Care Med 1998 Dec;158(6):1936-44.

(4) Laitinen A, Altraja A, Kampe M, Linden M, Virtanen I, Laitinen LA. Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. Am J Respir Crit Care Med 1997 Sep;156(3 Pt 1):951-8.

(5) Chakir J, Shannon J, Molet S, Fukakusa M, Elias J, Laviolette M, et al. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. J Allergy Clin Immunol 2003 Jun;111(6):1293-8.

(6) Huang J, Olivenstein R, Taha R, Hamid Q, Ludwig MS. Enhanced proteoglycan deposition in the airway wall of atopic asthmatics. Am J Respir Crit Care Med 1999;160:725-9.

(7) Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. Am J Respir Crit Care Med 2003 May 15;167(10):1360-8.

(8) Niimi A, Matsumoto H, Takemura M, Ueda T, Chin K, Mishima M. Relationship of airway wall thickness to airway sensitivity and airway reactivity in asthma. Am J Respir Crit Care Med 2003 Oct 15;168(8):983-8.

(9) Iozzo RV. Matrix proteoglycans: from molecular design to cellular function. Annu Rev Biochem 1998;67:609-52.

(10) Ruoslahti E, Yamaguchi Y. Proteoglycans as modulators of growth factor activities. Cell 1991 Mar 8;64(5):867-9.

(11) Roberts CR. Is asthma a fibrotic disease? Chest 1995 Mar;107(3 Suppl):111S-7S.

(12) Redington AE, Roche WR, Holgate ST, Howarth PH. Co-localization of immunoreactive transforming growth factor-beta 1 and decorin in bronchial biopsies from asthmatic and normal subjects. J Pathol 1998 Dec;186(4):410-5.

(13) de Kluijver J., Schrumpf JA, Evertse CE, Sont JK, Roughley PJ, Rabe KF, et al. Bronchial matrix and inflammation respond to inhaled steroids despite ongoing allergen exposure in asthma. Clin Exp Allergy 2005 Oct;35(10):1361-9.

(14) Moreno RH, Hogg JC, Pare PD. Mechanics of airway narrowing. Am Rev Respir Dis 1986 Jun;133(6):1171-80.

(15) McParland BE, Macklem PT, Pare PD. Airway wall remodeling: friend or foe? J Appl Physiol 2003 Jul;95(1):426-34.

(16) Pare PD. Airway hyperresponsiveness in asthma: geometry is not everything! Am J Respir Crit Care Med 2003 Oct 15;168(8):913-4.

(17) Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. Am J Respir Crit Care Med 2000;162(6):2341-51.

(18) Pepe C, Foley S, Shannon J, Lemiere C, Olivenstein R, Ernst P, et al. Differences in airway remodeling between subjects with severe and moderate asthma. J Allergy Clin Immunol 2005 Sep;116(3):544-9.

(19) de Medeiros MM, da Silva LF, dos Santos MA, Fernezlian S, Schrumpf JA, Roughley P, et al. Airway proteoglycans are differentially altered in fatal asthma. J Pathol 2005 Sep;207(1):102-10.

(20) Westergren-Thorsson G, Chakir J, Lafreniere-Allard MJ, Boulet LP, Tremblay GM. Correlation between airway responsiveness and proteoglycan production by bronchial fibroblasts from normal and asthmatic subjects. Int J Biochem Cell Biol 2002 Oct;34(10):1256-67.

(21) Ludwig MS, Ftouhi-Paquin N, Huang W, Page N, Chakir J, Hamid Q. Mechanical strain enhances proteoglycan message in fibroblasts from asthmatic subjects. Clin Exp Allergy 2004 Jun;34(6):926-30.

(22) Lambert RK, Wiggs BR, Kuwano K, Hogg JC, Pare PD. Functional significance of increased airway smooth muscle in asthma and COPD. J Appl Physiol 1993 Jun;74(6):2771-81.

(23) Wiggs BR, Hrousis CA, Drazen JM, Kamm RD. On the mechanism of mucosal folding in normal and asthmatic airways. J Appl Physiol 1997 Dec;83(6):1814-21.

(24) Macklem PT. A theoretical analysis of the effect of airway smooth muscle load on airway narrowing. Am J Respir Crit Care Med 1996 Jan;153(1):83-9.

(25) Bramley AM, Roberts CR, Schellenberg RR. Collagenase increases shortening of human bronchial smooth muscle in vitro. Am J Respir Crit Care Med 1995 Nov;152(5 Pt 1):1513-7.

(26) Todorova L, Gurcan E, Miller-Larsson A, Westergren-Thorsson G. Lung fibroblast proteoglycan production induced by serum is inhibited by budesonide and formoterol. Am J Respir Cell Mol Biol 2006 Jan;34(1):92-100.

(27) Al Jamal R, Roughley PJ, Ludwig MS. Effect of glycosaminoglycan degradation on lung tissue viscoelasticity. Am J Physiol Lung Cell Mol Physiol 2001 Feb;280(2):L306-L315.

(28) Fust A, LeBellego F, Iozzo RV, Roughley PJ, Ludwig MS. Alterations in lung mechanics in decorin-deficient mice. Am J Physiol Lung Cell Mol Physiol 2005 Jan;288(1):L159-L166.

(29) Hirst SJ, Twort CH, Lee TH. Differential effects of extracellular matrix proteins on human airway smooth muscle cell proliferation and phenotype. Am J Respir Cell Mol Biol 2000 Sep;23(3):335-44.

(30) Johnson PR, Burgess JK, Underwood PA, Au W, Poniris MH, Tamm M, et al. Extracellular matrix proteins modulate asthmatic airway smooth muscle cell proliferation via an autocrine mechanism. J Allergy Clin Immunol 2004 Apr;113(4):690-6.

FIGURE LEGENDS

Figure 1 Immunohistochemical staining for biglycan (A,B,D) and lumican (C,) in airway biopsies of patients with severe (A,C) and moderate (B) asthma, and a control subject (D). Note that in the biopsy specimens from severe asthmatic patients (A,C), biglycan and lumican immunostaining was sparse within the smooth muscle layer (short arrow) but prominent within the sub-epithelial layer (long arrow). In the biopsy specimen stained for biglycan from the moderate asthmatic patient (B), biglycan deposition was evident throughout both the smooth muscle (short arrow) and sub-epithelial (long arrow) layers. Biglycan immunostaining was relatively absent from the smooth muscle layer in the biopsy sample from the control subject (short arrow, D). Magnification x 200.

Figure 1 revised

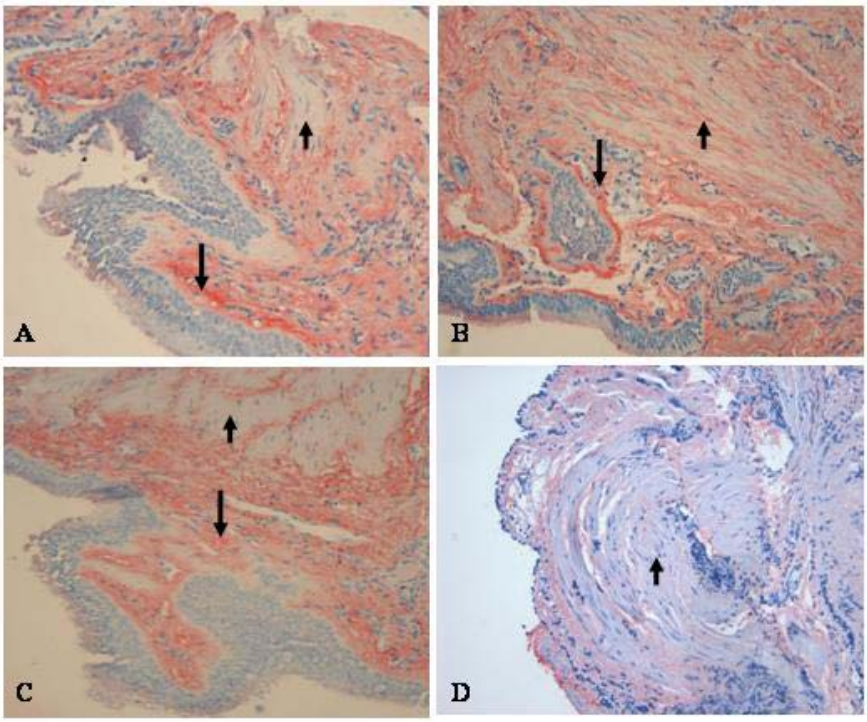

Figure 2 Area of positive staining in the subepithelial layer standardized for basement membrane length squared (A/bm^2) for biglycan, lumican, versican and decorin in biopsy specimens from severe and moderate asthmatic patients, and control subjects. Values are mean ± standard error. #, $p < 0.05$ vs control subjects.

Figure 2 revised

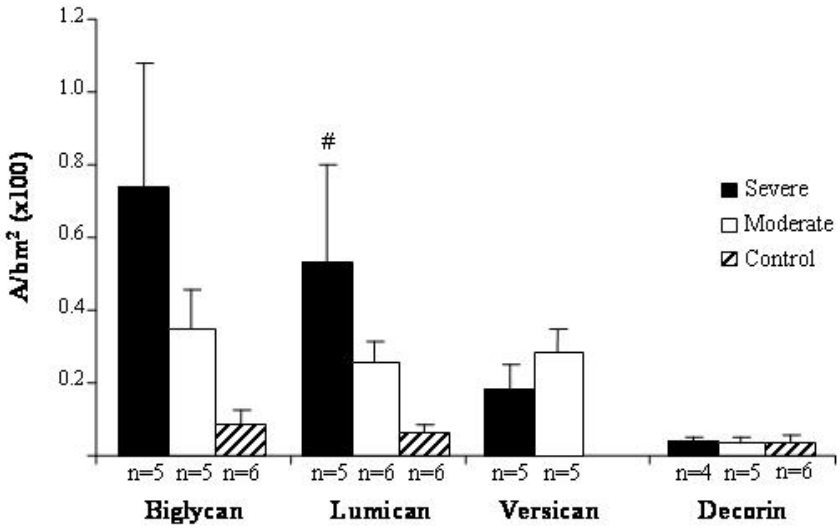

Figure 3 Percentage area of smooth muscle layer staining positive for biglycan, lumican, versican and decorin in biopsy specimens from severe and moderate asthmatic patients, and control subjects. Values are mean ± standard error. **, $p< 0.01$; *, $p < 0.05$ vs severe asthmatic patients. ##, $p < 0.001$ vs control subjects.

Figure 3 revised

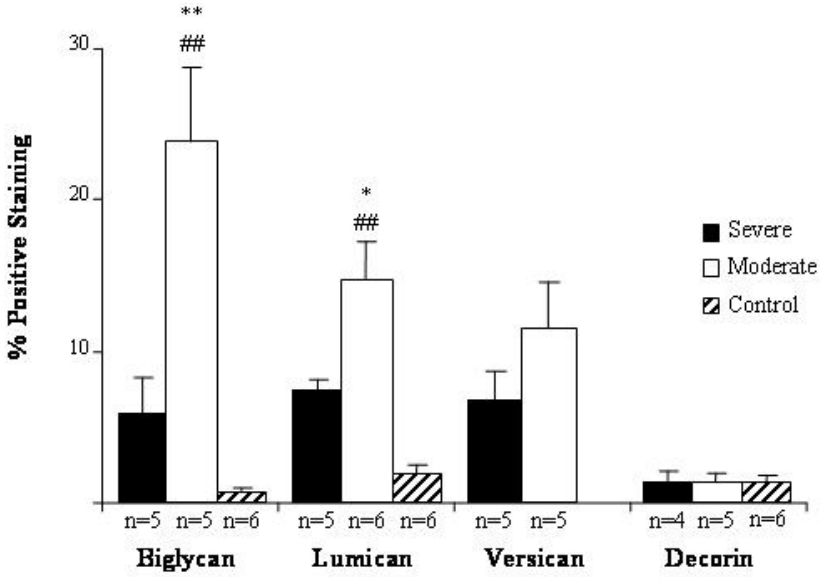

Table 1 Patient characteristics and spirometry

	GENDER	AGE	FEV_1	FEV_1(%pred)	FEV_1/FVC	Smoking (pk yrs)
Severe						
1	F	47	3	114	82	5
2	M	45	2	51	66	15
3	F	36	2.9	88	66	5
4	M	52	1.4	41	40	0
5	F	47	0.8	34	47	0
6	F	53	2.4	94	80	0
7	F	59	1	48	73	0
8	M	66	1.2	44	76	20
9	M	51	3.6	92	67	0
10	M	53	1.9	55	54	0
11	M	55	1.9	58	66	20
12	F	40	1.0	33	47	0
13	M	22	3.8	81	76	2
14	M	50	2.5	58	89.8	30
Mean		**48**	**2.1**	**63.6**	**66.4**	
SE			**0.3**	**6.8**	**3.9**	
Moderate						
1	M	49	2.5	67	70	5
2	F	50	2.2	82	72	3
3	F	61	2	100	76	20
4	F	55	2.1	84	72	0
5	M	55	3.1	77	78	10
6	F	23	2.9	95	73	0
7	M	42	3.1	78	62	12
8	M	60	2.5	74	63	0
9	F	47	2.5	85	79	2
10	M	49	4.4	119	82	0
11	M	55	5.2	121	74	0
12	M	39	2.1	78	71	0
13	M	50	2.7	77	77	17
Mean		**49**	**2.9**	**87.5**	**73.0**	
SE			**0.3**	**4.7**	**1.6**	
Control						
1	M	29	5.68	125	83	0
2	M	43	4.7	125	85	0
3	F	63	1.95	81	69	0
4	F	22	3.38	107	81	0
5	F	20	3.31	100	95	0
6	M	28	4.7	121	88	0
Mean		**34**	**3.96**	**109.8**	**83.5**	
SE			**0.5**	**7.1**	**3.5**	