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M. L. D'Antoni, C. Torregiani, P. Ferraro, M.-C. Michoud, B. Mazer, J. G. Martin and M. S. Ludwig

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Airway smooth muscle dynamics: a common pathway of airway obstruction in asthma

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Lung inflammation and vascular remodeling after repeated allergen challenge detected noninvasively by MRI

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Airway remodeling in allergen-challenged Brown Norway rats: distribution of proteoglycans

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Pini, Laura, Chiara Torregiani, James G. Martin, Qutayba Hamid, and Mara S. Ludwig. Airway remodeling in allergen-challenged Brown Norway rats: distribution of proteoglycans. *Am J Physiol Lung Cell Mol Physiol* 290: L1052–L1058, 2006. First published December 30, 2005; doi:10.1152/ajplung.00122.2005.—Proteoglycans (PG) have important effects on the mechanical properties of tissues and the phenotype of various structural cells. Little is known about changes in PG deposition in the airways in animal models of asthma. We studied changes in PG in the airway wall of Brown Norway rats sensitized to ovalbumin (OA) and exposed to repeated OA challenge. Control (Sal) animals were sensitized and challenged with saline. After the 3rd challenge, animals were killed and lungs fixed in formalin. Tissue sections were incubated with antibodies to the small, leucine-rich PG, decorin, and biglycan and collagen type I. Airways were classified according to basement membrane perimeter length (≤ 0.99 , 1–2.99, and ≥ 3 mm). Decorin, biglycan, and collagen type I were increased in the airways of OA vs. Sal rats. Remodeling was most prominent in central airways. The distribution of PG differed with respect to the subepithelial vs. airway smooth muscle (ASM) vs. adventitial layer. Whereas biglycan was readily detected within the ASM, decorin and collagen were detected outside the ASM and especially in the adventitial layer. Differences in the distribution of these molecules within the layers of the airway wall may reflect their specific functional roles.

decorin; biglycan; collagen

AIRWAY REMODELING IN ASTHMA is characterized by structural changes and thickening of the airway wall. A number of studies in both human asthmatics and animal models have reported an increase in extracellular matrix (ECM) characterized by excess interstitial collagen and fibronectin deposition (20, 22, 27). Less is known about changes in other ECM components, specifically, proteoglycans (PG). PG are macromolecules composed of a protein core and glycosaminoglycans side chains. These molecules subserve a number of important biologic functions, including regulation of ECM water balance, facilitation of cellular adhesion, proliferation and migration, and modulation of growth factor and cytokine activity (13). They are also important determinants of tissue mechanical properties (1, 10). The small leucine-rich PG gene family includes biglycan and decorin. These small PG possess similar core proteins characterized by the presence of adjacent leucine-rich regions. They interact with fibrillar collagen and influence the interaction of the collagen fibrils with other components of the ECM, thereby contributing to the maintenance of a normal extracellular milieu. Both biglycan and decorin bind transforming growth factor (TGF)- β and influence its bioavailability (11, 15). Recently, biglycan and decorin have been shown to

differentially affect lung fibroblast morphology, cytoskeleton, and migration (30).

The majority of studies of airway remodeling have focused on changes in matrix proteins in the subepithelium and reticular basement membrane. Changes in matrix deposition in the airway smooth muscle (ASM) and adventitial layer have not been as well defined, although Palmans et al. (22) reported, in chronically ovalbumin (OA)-challenged Brown Norway (BN) rats, that fibronectin was deposited primarily in the outer airway wall adjacent to the ASM. Alterations in ECM deposition in the ASM and adventitial layers could have important functional consequences, because of altered impedance to ASM shortening (4), effects on smooth muscle proliferation (14), and/or possible unlinking of airways and surrounding parenchymal attachments (17). Previous work from this laboratory in the BN rat model has shown that, after multiple OA challenges, both ASM mass and airway hyperresponsiveness are increased (6, 32); alterations in PG could contribute to these changes.

Although many studies have investigated remodeling in larger airways utilizing endobronchial biopsy (20, 25, 27), there is less information available on changes in the distal airways. Kraft (16) showed inflammation within the alveolar wall in human asthma, whereas Minshall et al. (19) showed evidence of increased cytokine expression in the peripheral airways. Palmans et al. (22), in their investigation of the BN rat model of chronic OA challenge, reported increases in fibronectin and collagen deposition in airways of all sizes. Whether PG are altered in this model is not known.

A number of studies using endobronchial biopsy to sample the large airways have reported increases in the PG, lumican, decorin, biglycan, and versican, in the airway subepithelial layer in atopic, mild asthmatic patients (9, 12, 25). Roberts (26), in autopsy studies of patients dying of fatal asthma, showed deposition of versican within the ASM layer. However, no studies have systematically compared the deposition of these molecules within the different layers of the airway wall or in central vs. more peripheral airways. To investigate these questions, we utilized the BN rat model of repeated OA exposure (29, 32).

METHODS

Animal Model

Male BN rats, 8–10 weeks old, weighing ~ 200 g, were sensitized by a single subcutaneous injection of 1 mg of OA (Sigma, Oakville, Ontario, Canada) and 100 μ g of aluminum hydroxide; Bordetella pertussis (5×10^8 heat-killed bacilli) was given intraperitoneally (ip)

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as an adjuvant. Challenged rats ($n = 6$) received aerosols of OA at 5-day intervals, on *days 14, 19, and 24* after sensitization. Controls rats ($n = 5$) were challenged with saline (Sal). Rats were sedated with xylazine (7 mg/kg ip) and anesthetized with pentobarbital sodium (30 mg/kg ip). Orotracheal intubation was performed with polyethylene tubing (PE 240, 6 cm in length). The tip of the tracheal tube was inserted into a Plexiglas box into which aerosols of OA (5% wt/vol) were delivered via a micromist nebulizer (Hudson RCI; Teleflex Medical, Temecula, CA), which delivers particles with a mean aerosol diameter of 2.05 μm . OA or Sal was administered for 5 min. 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 approved the protocol.

Immunohistochemistry

Tissue preparation and immunostaining. Two days after the last challenge, animals were killed by exsanguination, and lungs were removed. Lungs were fixed by intratracheal perfusion of 10% formalin at a constant pressure of 25 cmH_2O for 24 h. Lungs were embedded in paraffin, and sagittal sections (5 μm thick) were cut. Sections were incubated overnight with primary antibodies: rabbit polyclonal anti-mouse antibody for decorin (8) (1:1,000 dilution), rabbit polyclonal anti-human antibody for biglycan (28) (1:500 dilution) (both have been shown to cross-react with rat PG and were generous gifts of P. Roughley, Shriners Hospital, McGill University), or rabbit polyclonal anti-rat antibody for collagen type I (1:500 dilution, Cedarlane Laboratories, Hornby, Ontario, Canada). The secondary antibody applied was swine antibody to rabbit immunoglobulin (1:100 dilution; DAKO, Mississauga, Ontario, Canada). Streptavidin-alkaline phosphatase (1:200 dilution, DAKO) was used for detection. All immunostaining was developed with Fast Red (Sigma, Montreal, QC, Canada). Slides were counterstained with Gill II hematoxylin. Slides were examined under an Olympus light microscope (model BX50; Carson Group, Markham, Ontario, Canada). Positive staining appeared red under bright-field illumination. Control sections were processed in the absence of primary antibody.

PG immunolocalization by fluorescent microscopy. Excised lungs were fixed at a transpulmonary pressure of 25 cmH_2O with optimal cutting temperature medium and snap frozen in isopentane cooled in liquid nitrogen. Frozen sections were cut in 5- μm -thick sections with a cryostat and fixed in acetone-methanol. Sections were blocked by incubation with universal blocking solution for 30 min, followed by incubation with 10% normal goat serum. Slides were then rinsed with Tris-buffered saline (TBS) and incubated overnight at 4°C with mouse anti-human antibody for α -smooth muscle actin (α -SMA) (1:1,000 dilution, Sigma) and antibodies for biglycan or decorin. After being washed with TBS, sections were incubated with secondary antibody (1:1,000), 488 Alexa Fluor goat antibody to mouse immunoglobulin (α -SMA) (Molecular Probes, Burlington, Ontario, Canada), and 546 Alexa Fluor goat antibody to rabbit immunoglobulin, (PG) (Molecular Probes). After being washed with TBS, sections were sealed with crystal mount and viewed under an Olympus fluorescence microscope (model BX51).

Staining for eosinophils with major basic protein. Frozen sections from OA- and Sal-challenged rats were stained with major basic protein (MBP) to identify eosinophils in the airway wall. In brief, frozen slides were blocked with universal blocking solution and then incubated overnight at 4°C with primary antibody to MBP (anti-human, 1:30 dilution; a generous gift of Dr. R. Moqbel). After being washed with TBS, slides were incubated with secondary antibody (polyclonal rabbit, anti-mouse 1:60, DAKO). Slides were again washed with TBS and then incubated with mouse APAAP (1:60, DAKO), and the reaction was visualized via addition of Fast Red (Sigma). Slides were counterstained with Gill II hematoxylin, washed, dipped in lithium carbonate, rinsed again, and then sealed in crystal

mount. Sections were dried overnight at 37°C. Slides were examined with an Olympus microscope (model BX51); positive cells appeared red under bright-field illumination.

Morphometric Analysis

Microscopic images at a magnification of $\times 200$ were captured with commercial software (Image-Pro Plus, Silver Spring, MD). Only airways cut in transverse section, defined as a minimum to maximum internal diameter >0.33 , were examined (29). All airways from all animals that met these criteria were included in the analysis. The following measurements were made: basement membrane perimeter (Pbm); internal airway lumen (LuB) defined by the inner border of the epithelium; external area of the airway wall (Ae) defined by the external border of the adventitial layer; and total area of the airway wall [airway wall thickness (WA_T)] calculated as the difference between Ae and LuB. Lines were drawn with a digital pen (Wacom), and values were calculated with SigmaScan (Jandel Scientific, Corte Madera, CA). Airways were divided into three groups according to Pbm, small: $\text{Pbm} \leq 0.99$ mm; medium: 1.0–2.99 mm; and large: ≥ 3 mm. We quantified the amount of each ECM protein by tracing the area of positive staining established by determining a color threshold. The area of positive staining was standardized for airway size using Pbm^2 , as this scaled an area with an area.

Data Analysis

Comparison between OA and Sal within different-sized airway groups was performed by unpaired *t*-tests. ANOVA was used to perform comparisons among different-sized airways within OA and Sal groups. Linear regression was used to examine the correlation between staining for decorin and collagen in those airways in which staining was done in serial sections for the respective molecules. Linear regression by the least-squares technique was also applied to determine the relationship between WA_T and Pbm^2 in both OA and Sal groups. *t*-Test was used to determine differences in the slopes of the regression lines between OA and Sal for the three different-sized airway groups. Values of $P < 0.05$ were considered statistically significant. Values are means $\pm$ SE.

RESULTS

Biglycan, decorin, and collagen I immunoreactivity was readily identified in the airways of the rats sensitized and repeatedly challenged with OA as well as those sensitized and repeatedly challenged with Sal (Fig. 1, A–F). Staining for all proteins was more prominent in OA-challenged rats. Decorin and biglycan showed a different pattern of distribution. Biglycan was distributed within the ASM layer in both OA and Sal airways; decorin spared the smooth muscle bundles (see *insets* Fig. 1, A and C). Collagen seemed to be distributed in a similar manner to decorin. All proteins were detected in the subepithelial and adventitial layer; minimal positive staining was observed within the epithelium. Staining for eosinophils was carried out in frozen sections (Fig. 1, G and H). (The MBP antibody used required frozen tissue; lung morphology is not as well preserved as in formalin-fixed tissue.) Airway and perivascular eosinophilia was more prominent in OA-challenged rats compared with Sal control rats.

Quantification of the area of positive staining for biglycan, decorin, and collagen I is shown in Fig. 2. Biglycan immunostaining was increased in small and large airways in OA-challenged rats compared with the Sal-challenged rats ($P < 0.05$) (Fig. 2A). The area of positive staining for decorin was significantly increased in medium-sized airways in OA-challenged rats compared with the Sal-challenged rats ($P < 0.05$)

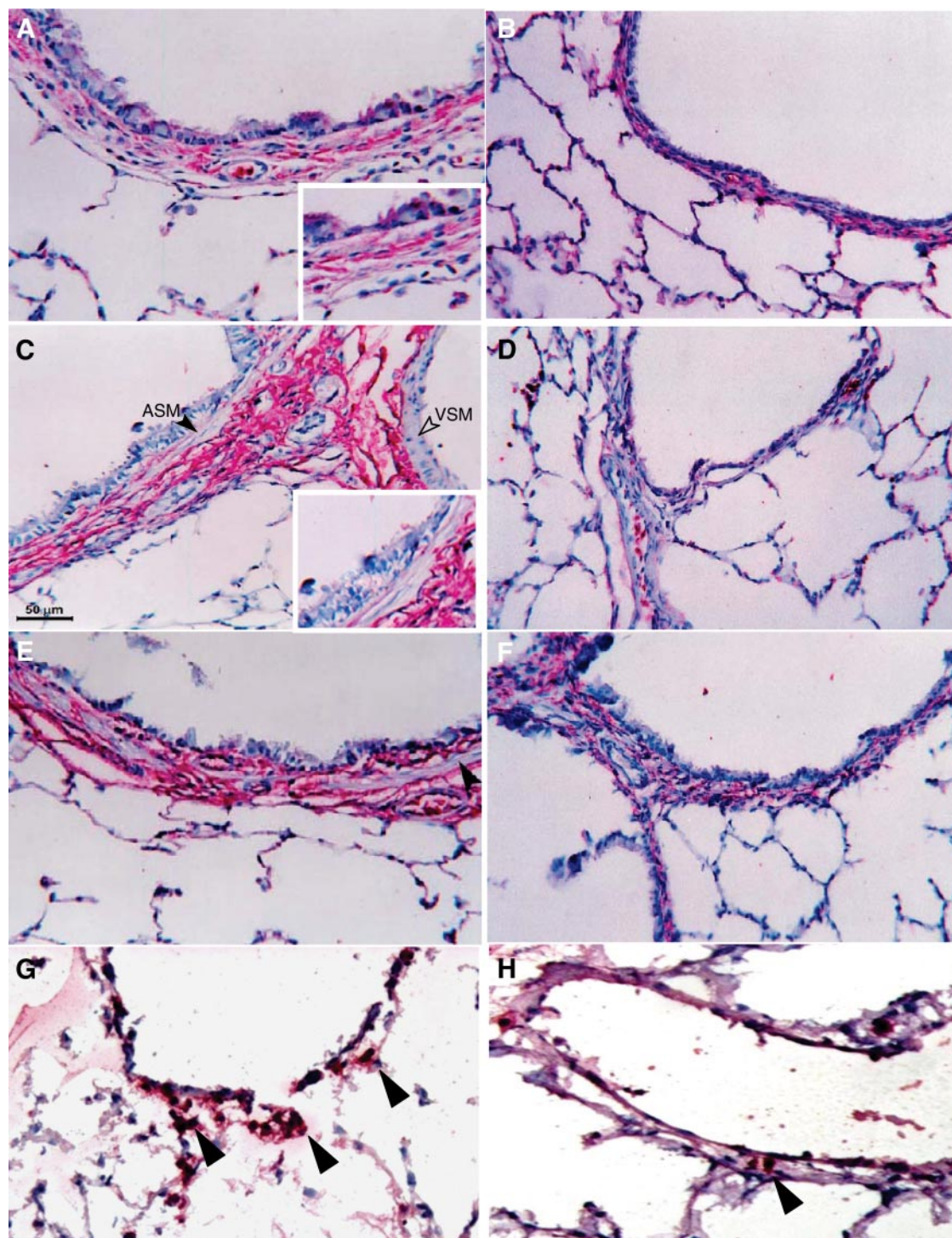


Fig. 1. Representative photomicrographs of immunostaining in the airway wall of ovalbumin (OA)-challenged (A, C, E, G) and saline (Sal) control rats (B, D, F, H). Staining of large airways with primary antibody for biglycan (A, B), decorin (C, D), and collagen type I (E, F). Positive staining for biglycan was present within the smooth muscle layer (A, see *inset*), whereas decorin staining occurred around the smooth muscle bundles (C, see *inset*). G and H show staining of small airways with antibody to major basic protein. Eosinophils (indicated by arrowheads) are more prominent in the airway wall of the OA-challenged rat. ASM, airway smooth muscle; VSM, vascular smooth muscle.

(Fig. 2B). The area of positive staining for collagen I was significantly increased in the large airways in OA-challenged rats compared with the Sal-challenged rats ($P < 0.01$) (Fig. 2C). Although the other comparisons did not achieve statistical significance, there was a trend toward greater positive staining

for all of the ECM molecules in all sized airways in the OA-challenged rats. We also compared the area of positive staining among the different-sized airways for the OA-challenged rats. The large airways had the greatest amount of biglycan compared with the medium and small airways (Fig.

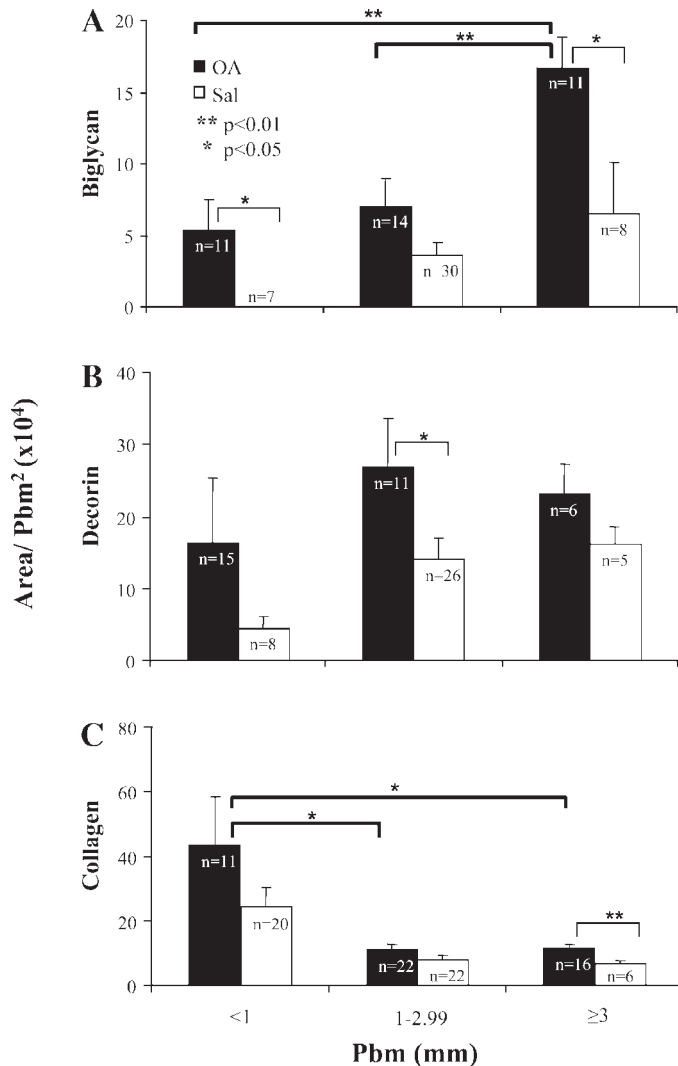


Fig. 2. Area of immunostaining for biglycan (A), decorin (B), and collagen I (C) in OA (solid bars) and Sal (open bars) rats in the 3 groups of airways. A, area; Pbm, basement membrane perimeter. Thin lines represent comparisons between airways from OA- and Sal-challenged rats; thick lines represent comparisons among small, medium, and large airways in OA-challenged animals. *n*, Total number of airways examined in each group. ***P* < 0.01; **P* < 0.05.

2A), whereas the amount of collagen was increased in the small airways compared with the medium and large airways (Fig. 2C).

In airways in which we had immunostaining for decorin and collagen in serial sections, we performed linear regression analysis to determine whether the amounts of collagen and decorin were correlated (Fig. 3). There was no significant correlation between staining for the two ECM molecules in the airways sampled (*n* = 14 airways).

There was a significant relationship between WA_T and the size of the airway as defined by Pbm^2 in both OA and Sal airways ($r = 0.85$ and 0.71 , respectively, $P < 0.01$) (Fig. 4). WA_T in the medium and large airways was increased in OA vs. Sal airways, as the slopes of the two regression lines were significantly different in these two subgroups ($P < 0.05$).

To better characterize the distribution of PG in relationship to the smooth muscle within the airway wall, we used fluo-

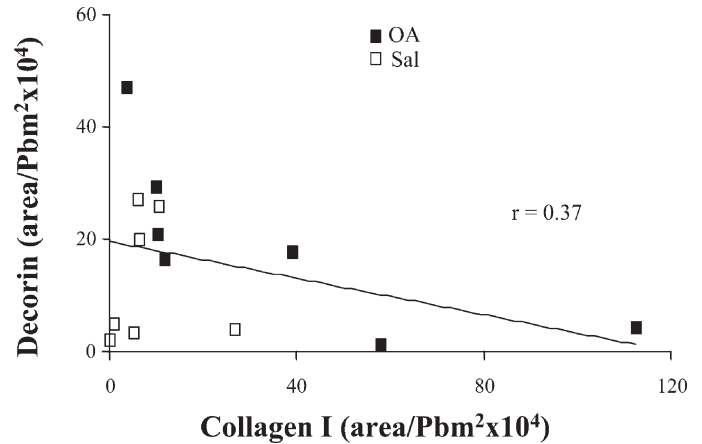


Fig. 3. Relationship between positive staining for decorin and collagen I in airways from OA (■)- and Sal-challenged (□) rats. Linear regression was not significant ($r = 0.37$). Only those airways in which both proteins were stained on serial sections were included in the analysis.

cent probes to perform double immunostaining for α -SMA and the PG of interest. As suggested by standard immunohistochemical staining, biglycan and decorin showed different patterns of deposition. Biglycan was distributed primarily within the ASM layer (Fig. 5 A, C, and E), whereas decorin was present around the ASM and was very prominent in the adventitial layer (Fig. 5, B, D, and F). This was particularly evident in serial sections of the same airway, stained with α -SMA and biglycan and α -SMA and decorin, respectively (Fig. 5, G and H).

DISCUSSION

The results of our study show that PG were increased in the airway wall of sensitized BN rats repeatedly challenged with OA. Although remodeling occurred in all sized airways, the predominant effect was seen in the larger airways. There was variation in the distribution of PG within the different layers of the airway wall; biglycan was prominent within the smooth muscle layer, whereas decorin was more prominent in the adventitial layer.

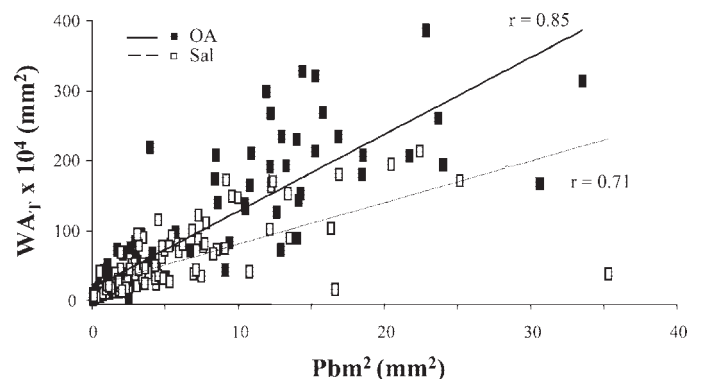


Fig. 4. Relationship between total wall area (WA_T) and basement membrane perimeter squared (Pbm^2) in airways from OA (■)- and Sal-challenged (□) rats. Linear regressions were significant for each group ($r = 0.85$ and 0.71 for OA and Sal, respectively, $P < 0.05$); the slopes of the 2 regression lines for the large and medium airway subgroups were significantly different from each other ($P < 0.05$).

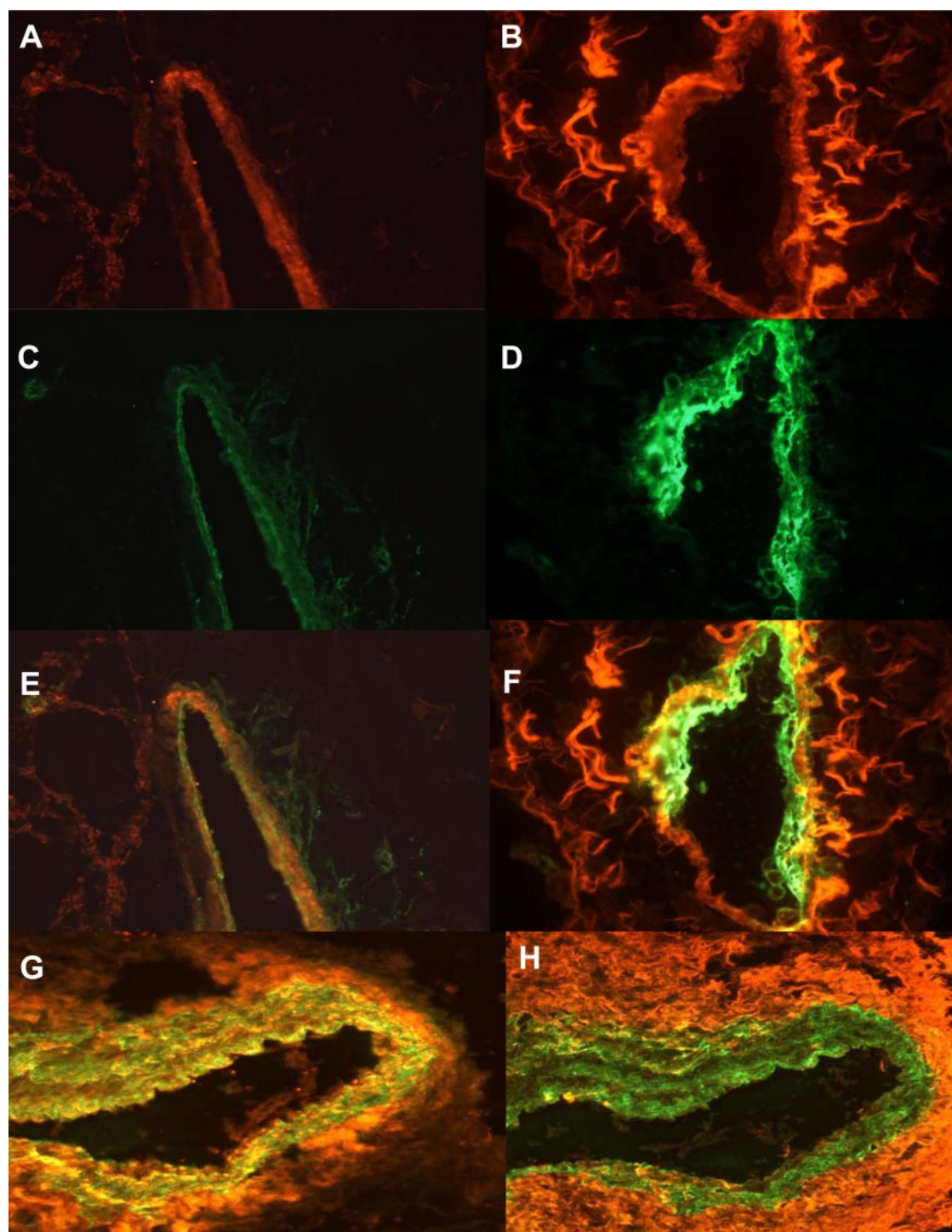


Fig. 5. Representative photomicrographs of double immunofluorescent staining. *A*, *C*, and *E* show staining of a medium-sized airway for biglycan (*A*, fluorescent red), α -smooth muscle actin (SMA) (*C*, fluorescent green) and the composite image (*E*). Biglycan was mainly localized in the smooth muscle layer. *B*, *D*, and *F* show staining of a small airway for decorin (*B*, fluorescent red), α -SMA (*D*, fluorescent green) and the composite image (*F*). Decorin staining occurred around the smooth muscle bundles, primarily in the adventitial layer. Staining of serial sections of the same airway with α -SMA and biglycan (*G*) and α -SMA and decorin (*H*) underscores the different patterns of PG deposition relative to the smooth muscle layer, even within the same airway.

Remodeling and changes in collagen and glycoproteins have been well described in the asthmatic airway wall in both humans and animal models (20, 22, 27). The absolute amount of collagen is increased in the subepithelial layer (20, 22); the specific subtypes of collagen upregulated include collagen

types I and III (5, 27). Characterization of alterations in PG is more recent. Using endoscopic techniques to sample large airways, we have shown increases in versican, lumican, and biglycan in the subepithelial layer of the airway wall in patients with mild asthma (9, 12). Redington and coworkers (25) have

reported substantial deposition of decorin in the reticular basement membrane. Roberts (26) reported prominent deposition of versican, biglycan, and decorin in the airway wall of patients dying from asthma or in whom asthma was a major contributor to death. In the current study, we extend these observations to an animal model of asthma, which permits a more careful characterization of the precise distribution of these molecules vis-à-vis the entire airway wall and the entire airway tree.

Significant increases in biglycan deposition were observed in both small and large airways, and significant increases in decorin deposition were observed in medium-sized airways in OA vs. Sal control animals (Fig. 2). We also characterized changes in collagen type I. Like Palmans et al. (22), who used a similar approach in chronically OA-challenged BN rats to characterize collagen deposition, collagen was increased in large airways in BN vs. control rats at ~4 wk postsensitization and challenge. In our study, however, the increases in collagen were generally less substantial than the increases in PG. The relatively greater changes in PG in this repeated challenge model may reflect the tendency of PG to be upregulated at an earlier time point in the remodeling process, establishing a "provisional matrix" into which collagen and elastic fibers may be subsequently laid down. This temporal sequence has been described for pulmonary fibrosis in both humans and animal models (2, 31). Moreover, in this study, airway eosinophilia was more prominent in the OA-challenged rats, as has been shown previously in this repeated challenge BN rat model (22). This suggests that acute inflammation was ongoing.

We also examined whether matrix protein deposition within the airways of OA-challenged animals differed as a function of airway size. Although large airways showed the greatest deposition of biglycan, the amount of collagen deposition was greatest in the smaller airways. These latter data are different from those reported by Palmans et al. (22) in their OA-challenged animals examined at 4 wk, although their animals received six challenges over the 4-wk period compared with the three challenges delivered to the animals in the current protocol.

Overall, our data demonstrated a relatively greater increase in matrix deposition in the larger airways in the OA-challenged animals. This can perhaps be explained by the method of OA challenge employed in the current protocol. Antigen was administered by aerosol inhalation. The aerosol particles were most likely to be deposited on the mucosa of the larger-sized airways (3). As this is the type of antigen challenge that typically occurs in asthmatic patients, we believe it is the most pertinent to study.

We also investigated the deposition of PG molecules within the various layers of the airway wall. Immunohistochemical studies showed that there were different patterns of PG distribution; biglycan seemed to be prominent within the subepithelial and smooth muscle layer, whereas decorin spared the smooth muscle layer and was most evident in the adventitial layer. To further clarify the distribution of PG in relationship to ASM, we performed double immunostaining studies using α -SMA antibody to identify ASM. We observed localization of biglycan and α -SMA within the same layer of the airway wall; decorin was prominent outside the α -SMA-positive layer, especially in the airway adventitia (Fig. 5). The data describing the distribution of decorin are similar to those reported by Roberts (26) in airway walls of patients dying of asthma, in

whom they describe decorin prominence in the airway adventitia and septal structures. This pattern of distribution in patients with longstanding disease may reflect the importance of decorin as a regulator of collagen fibril formation. In areas where collagen deposition is prominent, decorin should be present. However, we were unable to document a significant correlation between decorin and collagen deposition in airways in which staining for both ECM molecules was performed (Fig. 3). This may reflect that, in this model, the airway remodeling process was in a relatively early stage.

The close association of biglycan with the smooth muscle layer is perhaps more surprising. Potter-Perigo et al. (24) have recently reported in human cell culture studies that biglycan was preferentially produced by vascular smooth muscle cells, whereas decorin was produced by bronchial smooth muscle cells. The pattern of distribution we observed suggests that cell secretion and accumulation of PG within the airway wall may be altered in asthmatic disease.

These data also raise the question of the functional impact of PG molecules on the surrounding cellular environment. PG are ECM proteins that subserve a number of important biological functions. They interact with various cytokines and growth factors, influence biomechanical behavior of the tissues, and affect cell adhesion and migration (13). Decorin and biglycan interact with TGF- β , a cytokine known to play an important role in the airway fibrosis observed in asthma (20, 25). A recent study by Tufvesson and Westergren-Thorsson (30) describes the effects of biglycan and decorin on lung fibroblast phenotype. Both PG induced comparable changes in fibroblast morphology and stress fiber formation. However, important differences were noted in the amount of α -SMA induced, a finding that could have potential implications for differential effects of decorin and biglycan on ASM phenotype. Moreover, decorin and biglycan differentially upregulated mRNA for proteins involved in cell-matrix interactions, cell-cell interactions, and intracellular signaling. In vascular smooth muscle, the large PG, versican, is required for normal cell proliferation (7). A recent study by Johnson et al. (14) reported that ECM proteins secreted by asthmatic ASM cells enhanced ASM cell proliferation. Excess biglycan within the smooth muscle layer in the airway wall of OA-challenged animals may have the capacity to augment the smooth muscle hypertrophy previously reported in this model (6, 32).

The distribution of PG molecules within the various layers of the airway wall in the asthmatic model could also have implications for airway function from a more mechanical perspective. It has consistently been shown in the OA-challenged, BN rat model that multiple OA challenges lead to enhanced airways responsiveness (22, 32). Data from our laboratory demonstrate that PG influence both airway mechanics and the viscoelastic behavior of the lung tissues (1, 10). Specific enzymatic degradation of glycosaminoglycan side chains altered lung parenchymal strip viscoelastic properties (1). Both airway resistance and tissue compliance measured in vivo were altered in mice deficient in the small PG, decorin (10). Hence, excess deposition of PG within the airway wall could potentially affect airway mechanical properties and airway responsiveness (21). The distribution of PG deposition within the smooth muscle layer of the airway wall could also have an impact on the impedance to ASM shortening during induced constriction (4, 23). Finally, changes in PG deposition

in the adventitial layer could have an effect on airway parenchymal interdependence. The airway wall could become unlinked from the modulating effects of the surrounding parenchymal attachments, permitting airways to constrict relatively unimpeded (18). Alternately, excess deposition of PG could affect the tethering between the airways and parenchyma in such a way as to augment the impedance offered by the surrounding parenchymal attachments. Palmans and coworkers (22) reported excess fibronectin deposition in the outer wall layer of chronically OA-challenged BN rats and similarly postulated an impact on airway parenchymal interdependence and bronchial reactivity, although less is known about the biomechanical characteristics of fibronectin.

In conclusion, we have found that decorin, biglycan, and collagen type I are upregulated in the airway wall of sensitized BN rats exposed to repeated OA challenge; the greatest changes occurred in the larger airways. The pattern of distribution in the ASM and adventitial layer, of the two small PG studied, biglycan and decorin, differed. To better understand how changes in these molecules affect cell phenotype, studies on the effects of PG on the functional behavior of the adjacent structural cells are warranted. The BN rat model of allergic asthma provides a ready source of cells to further pursue these questions.

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