Airway proteoglycans are differentially altered in fatal asthma

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Abstract

It has been suggested that airway remodelling is responsible for the persistent airway obstruction and decline in lung function observed in some asthmatic patients. The small airways are thought to contribute significantly to this functional impairment. Proteoglycans (PGs) are important components of the extracellular matrix (ECM) in the lungs. Besides controlling biophysical properties of the ECM, they play important roles in the regulation of some cytokines. Increased subepithelial PG deposition in the airways of mild asthmatics has been reported. However, there are no data on the PG content in small airways in asthma. This study has compared the content and distribution of PGs in large and small airways of patients who died of asthma with those in control lungs. Immunohistochemistry and image analysis were used to determine the content of lumican, decorin, biglycan, and versican in large (internal perimeter >6 mm) and small (internal perimeter ≤6 mm) airways of 18 patients who had died of asthma (A) and ten controls (C). The results were expressed as PG area (μm²)/epithelial basement membrane length (μm). The main differences between asthmatics and controls were observed in the small airways. There was a significant decrease in decorin and lumican contents in the external area of small airways in asthmatics (decorin: A 1.05 ± 0.27 μm, C 3.97 ± 1.17 μm, p = 0.042; lumican: A 1.97 ± 0.37 μm, C 5.66 ± 0.99 μm, p = 0.002). A significant increase in versican content in the internal area of small and large airways in asthmatics was also observed (small: A 7.48 ± 0.84 μm, C 5.16 ± 0.61 μm, p = 0.045; large: A 18.38 ± 1.94 μm, C 11.90 ± 2.86 μm, p = 0.028). The results show that PGs are differentially expressed in the airways of fatal asthma and may contribute to airway remodelling. These data reinforce the importance of the small airways in airway remodelling in asthma.

Keywords: asthma; proteoglycans; small airways; image analysis; autopsy, remodelling

Introduction

A number of asthmatic subjects show evidence of persistent airway obstruction and decline of lung function with time [1]. It is believed that airway remodelling may be responsible for the persistent functional changes observed in these patients [2]. Several structural changes are related to airway remodelling in asthma: muscle hypertrophy/hyperplasia; mucus gland hyperplasia; and changes in the extracellular matrix (ECM). All components of the lung ECM (collagen, elastic fibres, proteoglycans, and glycoproteins) have been shown to be potentially altered in asthma [3,4]. It has been proposed that changes in the epithelial–mesenchymal trophic unit due to epithelial injury in asthma are responsible for increased secretion of pro-fibrogenic cytokines such as TGF-β, myofibroblast proliferation, and subepithelial deposition of ECM components [5].

It has also been suggested that small airway alterations may contribute significantly to the functional impairment in asthma, especially in the most severely affected patients [6–8]. The peripheral airways are thought to be the major site of airway obstruction in asthmatic patients [9]. Airway remodelling has been largely investigated in the large airways in asthma and there are still relatively scarce data on the small airways.

Proteoglycans (PGs) are major components of the ECM and present many different biological functions. PGs have been implicated in maintenance of the biophysical properties of many tissues, in the assembly of collagen fibrils, and in tissue regulation of water balance; they influence cell migration, act as growth
developers, and influence vessel permeability [10]. It has also been proposed that PGs play important roles in the regulation of some cytokines, such as in TGF-β action [11].

Few studies have investigated the role of PGs in airway remodelling in asthma. Increased deposition of lumican, biglycan, and versican has been shown in the subepithelial layer in the airways of mild atopic asthmatic subjects [12]. Versican and hyaluronan deposition has been reported in the airway walls of patients with fatal asthma [13]. However, there are no data on the PG content of small airways in asthma. The characterization of PG distribution along the respiratory tract of asthmatic airways may help in the understanding of the role of PGs in airway remodelling, particularly in small airways. For this purpose, we determined the PG content in large and small airways in patients who had died of asthma and compared it with that of non-asthmatic control subjects.

Materials and methods

This study was approved by the review board for human studies of the School of Medicine of the University of São Paulo (CAPPesp-FMUSP). The procedures followed in this work were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983.

Autopsies were performed in our autopsy service on 18 patients who had died of ‘status asthmaticus’ between January 1996 and December 2000. The patients submitted to autopsy in this service come from different hospitals or from their homes. In many instances, clinical charts are absent. An interview with a first-degree relative was made prior to autopsy and information concerning main diseases was obtained. Inclusion criteria were (1) asthma history: all patients included in the study were known to be asthmatics and had died during an acute attack; and (2) pathological changes consistent with fatal asthma: all patients showed lung hyperinflation and hypersecretion, epithelial desquamation, mucosal oedema, and airway inflammation with or without eosinophils [14]. Exclusion criteria: patients were excluded when histological analysis showed signs of any other previously undiagnosed pulmonary disease.

Further clinical data (smoking habits, treatment history, medical follow-up, previous hospital admissions, and duration of the final crisis) were obtained by a questionnaire completed by first-degree relatives of the deceased at their homes.

Ten non-smoking individuals who had died as a consequence of non-pulmonary causes, with no previous pulmonary diseases, were selected as controls. Control subjects did not have a history of asthma. Control patients showed normal lungs at gross and microscopic examination.

Four pulmonary fragments from the peripheral and central areas of the lung were randomly collected from all patients. Tissue was fixed in 4% paraformaldehyde, routinely processed, and embedded in paraffin wax. Four-micrometre-thick sections were stained with haematoxylin and eosin (H&E) or submitted to immunohistochemistry.

For immunohistochemistry, sections were dewaxed and a 0.3% hydrogen peroxide in methanol solution was applied for 10 min. Decorin, biglycan, and lumican were identified using rabbit anti-human polyclonal antibodies that were characterized for specificity as described previously [15,16]. Versican was identified using a mouse anti-human monoclonal antibody (Seikagaku Co, Tokyo, Japan). Briefly, sections were incubated with 0.05 U/ml chondroitinase ABC (Sigma, Oakville, Ontario, Canada) for 1 h at 37 °C and then incubated with the primary antibody (1:4000 for biglycan, 1:250 for decorin, 1:800 for lumican, and 1:2000 for versican) in 1% BSA/PBS at room temperature for 60 min. As a secondary antibody, the horseradish peroxidase-conjugated anti-rabbit or antimouse EnVision system (DAKO, Glostrup, Denmark) was used. NovaRED (Vector, Burlingame, CA, USA) was used as a chromogen. The sections were counterstained with Mayer’s haematoxylin. For negative controls, the primary antibody was omitted from the staining. All of the sections were stained within one staining session using antibodies coming from one batch.

Transversely cut airways were defined as those showing a short/long diameter ratio greater than 0.6. They were classified based on their epithelial basement membrane (EBM) perimeter (Pi) as large airways (Pi > 6 mm), corresponding to small cartilaginous airways [17], and small airways (Pi ≤ 6 mm), corresponding to membranous bronchioles [17–23]. Each airway was subdivided into inner and outer areas [24]. The inner area was located between the epithelium and the internal smooth muscle border, including the EBM and the lamina propria. The outer area was located between the external smooth muscle border and the external limits of the airway, ie the lung parenchyma. In large airways, cartilage and submucosal glands may be interposed between the smooth muscle and the parenchyma; these structures were therefore excluded from the measurements.

PG content was determined using image analysis. Measurements of positive stained areas were performed with the software Image-Pro® Plus 4.1 for Windows® (Media Cybernetics, Silver Spring, MD, USA) on a personal computer connected to a digital camera coupled to a light microscope (Leica DMR, Leica Microsystems Wetzlar GmbH, Germany). We measured the area of PG positive staining corrected for EBM length in ten 400× randomly selected fields for each studied region in large airways and the entire circumference of two small airways. Selection of airway regions to be analysed as well as PG content analysis

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was performed by a blinded investigator. PG content was expressed as PG area (µm²)/EBM length (µm).

Statistical analysis was performed with the SPSS 10.0 software (SPSS, Chicago, IL, USA). Independent samples T-test after logarithmic data transformation was used to compare the content of each PG in asthmatic and control airways, as well as to compare the airway perimeters between asthmatic and control subjects. One-way ANOVA multiple comparisons followed by Tukey’s test after logarithmic data transformation were applied for comparison of the PG content among lung compartments in controls and asthmatics. Results were expressed as means ± standard error. Correlations were calculated using the Spearman rank test. The level of significance was set at p < 0.05.

Results

Subjects’ characteristics are shown in Tables 1 (asthmatics) and 2 (controls). Asthmatics’ median age was 39 years, ranging from 15 to 68 years, and ten of them were female. Controls’ median age was 46 years, ranging from 20 to 74 years, and nine of them were female. Among the asthmatic subjects, five were current smokers. All had been using inhaled β-agonists. Only three patients had been treated with corticosteroids: one of them received beclomethasone regularly plus oral steroids during exacerbations; one patient received only beclomethasone regularly; and the other received oral steroids during exacerbations.

All asthmatic patients had a macroscopic and histological picture compatible with asthma (hyperinflation, hypersecretion, epithelial desquamation, thickening of basement membrane, hypertrophic submucosal glands, hypertrophic smooth muscle, mucosal inflammation with or without eosinophils) and their deaths were ascribed to status asthmaticus by the pathologist. All control patients had normal lungs at gross and microscopic examination.

Adequate samples (lung tissue containing large and small transversely-cut airways) were available for 13 asthmatics and nine controls for decorin staining, 13 asthmatics and nine controls for biglycan, 15 asthmatics and seven controls for lumican, and 16 asthmatics and nine controls for versican. A total of 73 large and 113 small airways were measured. The mean perimeters of large asthmatic and control airways were 9.36 mm and 9.02 mm (range 7.81–10.80 mm and 7.06–12.88 mm), respectively, corresponding to subsegmental bronchi [25]. The mean perimeters of small airways for asthmatics and controls were 1.63 mm and 1.65 mm (range 0.33–2.76 mm and 1.01–4.12 mm), respectively, corresponding to small membranous bronchioles [25]. There was no significant difference in airway perimeter between asthmatics and controls.
Descriptive analysis

PG immunoreactivity showed similar patterns of distribution in both control lungs and asthmatic patients. There was no staining in epithelial or smooth muscle cells. The small PGs decorin, biglycan, and lumican showed similar patterns of staining. We observed variable, but usually strong, reactivity in the reticular basement membrane (RBM). These three small PGs were predominantly localized within the ECM around airways and blood vessels, with stronger reactivity in the outer area than in the inner area of the airway wall for decorin and biglycan. Furthermore, the three small PGs tended to present stronger staining in large airways compared with small airways. There was also positive staining in the ECM within airway smooth muscle cells and strong reactivity around cartilage plaques. In the alveolar parenchyma, these PGs had a similar and scattered pattern of staining in both alveolar ducts and alveolar walls. Versican showed weak staining in the RBM and strong and continuous immunoreactivity in the lamina propria of airway walls. Differently from the small PGs, it showed weak staining within the ECM around airways and blood vessels. The intensity of staining tended to be stronger in the inner area than in the outer area of the airways, with no differences between small and large airways. Versican was also observed in the matrix within smooth muscle cells in both airways and blood vessels. It showed weak reactivity around cartilage plaques and, in the alveolar parenchyma, it presented a similar pattern of staining to that observed for the small PGs. Figure 1 shows representative photomicrographs of PG staining in small airways of asthmatics and controls.

Quantitative analysis

Figure 2 shows the PG content in asthmatics and controls. Decorin (Figure 2A) and lumican (Figure 2B) were significantly decreased in the outer area of small airways in asthmatics compared with controls (decorin: $A = 1.05 \pm 0.27 \mu m$, $C = 3.97 \pm 1.17 \mu m$, $p = 0.042$; lumican: $A = 1.97 \pm 0.37 \mu m$, $C = 5.66 \pm 0.99 \mu m$, $p = 0.002$). No difference was observed in the other airway regions. There was no significant difference in biglycan content in any studied area between asthmatics and controls (Figure 2C). The inner areas of large and small airways showed a significantly higher versican content in asthmatics compared with controls (large airways: $A = 18.38 \pm 1.94 \mu m$, $C = 11.90 \pm 2.86 \mu m$, $p = 0.028$; small airways: $A = 7.48 \pm 0.84 \mu m$, $C = 5.16 \pm 0.61 \mu m$, $p = 0.045$) (Figure 2D).

Comparisons between different airway compartments showed that in both control and fatal asthma groups, the highest decorin content was observed in the outer area of large airways. Among control patients, there was a significant difference in decorin content between the outer area of large airways and the inner area of small airways ($p = 0.014$). Among asthmatic patients, both inner and outer areas of small airways presented a significant decrease in decorin content compared with the outer area of large airways ($p < 0.01$) (Figure 2A). Lumican content in controls was significantly decreased in the inner area of small airways compared with the outer areas of small and large airways ($p < 0.02$). Among asthmatic patients, both inner and outer areas of small airways showed a significantly decreased lumican content compared with inner and outer areas of large airways ($p < 0.03$) (Figure 2B). Both control and asthmatic patients showed a progressive rise in biglycan content towards the large airways. In controls, the inner area of small airways presented a decreased biglycan content compared with the three other studied areas ($p < 0.02$). In asthmatics, a significant decrease in biglycan content was observed in the inner area of small airways compared with the outer area of large airways ($p = 0.001$) (Figure 2C). Versican content was similar in all studied areas of control patients. Among asthmatic patients, the inner area of large airways showed the highest versican content, which was significantly different from all other studied regions ($p < 0.004$) (Figure 2D).

In order to investigate whether clinical data, such as duration of disease, duration of the final crisis, and age, could have had any influence on the PG content, we performed correlations between these clinical parameters and PG data. There were no significant correlations.

Discussion

In the present study, we have shown significant quantitative differences in PG content between control and asthmatic airways, which may contribute to airway remodelling in fatal asthma. Although all studied PGs tended to present a higher content in the large airways than in the small airways, the main differences between fatal asthma and controls were observed in the small airways, with a significant decrease in decorin and lumican content in the outer area and a significant increase in versican content in the inner area of small airways in fatal asthma. To our knowledge, this is the first study to determine PG content in small airways in asthma.

Differences in PG content were observed in the different airway layers and sizes. The functional consequences of airway remodelling are dependent on which layer in the airway wall is changed as well as on the composition and mechanical properties of the material that is altered [26]. In normal lungs, the area inside the smooth muscle provides resistance of the tissue to compression. The outer wall, especially in small airways, is directly attached to the lung parenchyma and is therefore crucial for maintaining lung tissue structure and for transmitting elastic forces. We therefore believe that airway compartmentalization certainly contributes to a better understanding of the
Figure 1. Immunohistochemical staining of asthmatic (right panels) and control (left panels) small airways with anti-decorin (A, B), anti-lumican (C, D), and anti-versican (E, F). Decorin and lumican contents are decreased in the outer area of small airways in asthmatics (B and D, respectively) compared with controls (A and C, respectively). Versican content is increased in the inner area of small airways in asthmatics (F) compared with controls (E). OA = outer area; IA = inner area; M = smooth muscle; L = lumen. Scale bar = 50 µm

structure–function relationship in a pathological condition such as asthma.

Decorin is a small leucine-rich PG with a protein core and one glycosaminoglycan chain (chondroitin sulphate/dermatan sulphate); it is distributed throughout most connective tissues and is likely to play important roles in organizing the extracellular matrix [27,28]. Decorin core protein binds to collagen and decreases the rate of collagen fibrillogenesis in vitro [29,30]. Decorin has also been shown to play important regulatory roles on TGF-β action [11]. In the airways, decorin and TGF-β share the same immunoreactivity distribution pattern [31]. Furthermore, TGF-β inhibits decorin synthesis by lung fibroblasts [32]. Since TGF-β is directly related to the amount of fibrosis in the lungs [33], it would be expected that a decreased decorin content in asthmatic airways would reflect a higher pro-fibrotic TGF-β level, which would lead to collagen deposition and airway remodelling. Insofar as decorin plays a negative feedback role in TGF-β production [11], the decrease in decorin content could in turn lead to increased...
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Figure 2. The graphs show proteoglycan content in all studied regions in asthmatic patients and controls. IS = inner area of small airways; OS = outer area of small airways; IL = inner area of large airways; OL = outer area of large airways. Boxes represent the means and bars represent 2 standard errors of mean. ∗p < 0.05 compared with controls. (A) Decorin: †p < 0.05 compared with IS; §p < 0.05 compared with IS and OS. (B) Lumican: †p < 0.05 compared with OS and OL; §p < 0.05 compared with IS and OS. (C) Biglycan: †p < 0.05 compared with OS, IL, and OL; §p < 0.05 compared with OL. (D) Versican: †p < 0.05 compared with IS, OS, and OL. Proteoglycan content is expressed as PG area (µm²)/EBM length (µm).

TGF-β activity. Determining collagen and TGF-β content in these airways would certainly improve our understanding of decorin–TGF-β–collagen interaction in airway remodelling.

Contrary to our finding of decreased decorin in small airways in fatal asthma, Ludwig et al have shown greater in vitro expression of decorin mRNA in fibroblasts from asthmatic patients than from non-asthmatics [34]. Westergren-Thorsson et al have also examined PG production by asthmatic fibroblasts and documented increased production of perlecan, versican, biglycan, and small heparan sulphate PGs, but not of decorin [32]. These conflicting results may be explained by differences in asthma phenotypes (mild asthma versus fatal asthma) and analysis of cell cultures [32,34] versus tissue (the present study), and suggest that the role of decorin in airway remodelling deserves further investigation.

Lumican is a small leucine-rich PG with a protein core and keratan sulphate chains. We have previously reported the expression of lumican in peripheral human lung tissue. In the distal lung, lumican is mainly observed in the ECM of vessel walls, but it is also observed in airway walls and in alveolar septa [35]. Although lumican density has been shown to be increased in the reticular basement membrane of mild asthmatics and decreased after anti-IL-5 treatment [36], we observed a decrease in lumican content in the small airways in fatal asthma. Similarly to decorin, lumican core protein binds to collagen and is involved in collagen fibril organization [37,38]. Lumican has also been shown to decrease the rate...
of collagen fibrillogenesis [39]. We expect that the decrease of lumican content in the small airways of asthmatic patients may, as hypothesized for decreased decorin, lead to increased collagen content and play a role in the mechanism of airway remodelling.

Increased versican deposition has been previously shown in asthmatic airways [12,13]. Versican is a large chondroitin sulphate PG with a protein core rich in glutamic acid, serine, and threonine residues. It controls fluid balance and affects tissue resilience. It has been shown that stimulation of arterial smooth muscle cells with TGF-β1 and platelet-derived growth factor (PDGF) increases the steady-state level of mRNA that hybridizes to versican cDNA [40]. It is likely that the increase in this PG content in asthma is related to the cytokine balance in the inflammatory process. Deposition of versican has been observed in other inflammatory lung pathologies, such as pulmonary fibrosis and granulomatous diseases. In such conditions, collagen synthesis takes place in a versican-rich matrix [41]. It has been proposed that versican may influence myofibroblast proliferation and collagen deposition in asthma [42]. The increase in versican content in the inner airway area of both large and small airways in asthma may increase the resistance of the tissue to compression and represent a protector effect, preventing airway deformation and collapse. Conversely, increased airway thickening by ECM deposition, internal to the smooth muscle, may amplify the narrowing effect produced by muscle shortening [42,43].

Although an increase in biglycan in the subepithelial layer has been described in mild asthmatics [12], we did not observe any difference between fatal asthma patients and controls regarding the content of this PG in any airway region. It is possible that this discrepancy is related to different assessment sites of biglycan content, since the subepithelial layer corresponds to the reticular basement membrane and excludes the lamina propria. Insofar as we did not measure the reticular basement membrane separately, it is not possible to compare our results with those previously reported by Huang et al [12]. Also, differences in biglycan content may again be related to differences in asthma phenotypes.

Although all of the patients studied died of asthma, we do not have detailed clinical information concerning allergic status, allergen exposure or pulmonary function tests, which have certainly limited the evaluation of the clinical implications of our results. Since clinical data are very heterogeneous in this group of patients, there is also a clear limitation in performing correlations between PG content and clinical data, and the absence of significant correlations needs to be interpreted with caution. Furthermore, the extent to which the results obtained with fatal asthma patients can be transposed to less severe asthma cases is unclear.

Considerable attention has been given to the distal lung in asthma, especially because of the potential association between small airway alterations and asthma severity [24,44]. The peripheral airways are thought to be the major site of airway obstruction in patients with asthma [9]. Although subepithelial fibrosis and thickening have been reported in large asthmatic airways due to excess deposition of collagen, laminin, fibronectin, and PGs [3,12], relatively little is known about structural remodelling of the distal airways. Carroll et al [25] have shown increased small airway thickness in both non-fatal and fatal asthma compared with controls. We observed that the small airways showed the major differences in PG content between fatal asthma and controls. The inflammation in the distal lung in asthma has been shown to be more severe than in large airways [24], which could, at least partially, contribute to our results. We have recently analysed the inflammatory profile of large and small airways in fatal asthma [45]. Interestingly enough, the most affected airway region in both studies was the outer area of small airways, which showed in the present study a significant decrease in decorin and lumican content. In the previous study, we identified this region as the main site of inflammatory changes in fatal asthma, with significant increases in eosinophil, mast cell, and lymphocyte content in asthmatics. We also observed that mast cells presented the highest cell content in the outer wall of small membranous bronchioles when compared with other airway regions. Mast cells secrete mediators that may have different effects on ECM components. It is likely that this cell plays an important role in distal lung remodelling in fatal asthma [46–49].

The distribution of interstitial PGs in normal and emphysematous lungs was previously reported by van Straaten et al [50]. The authors showed decreased decorin and biglycan staining in the peribronchiolar area in lung tissue from patients with severe emphysema, suggesting that the diminished presence of these PGs may be part of the parenchymal abnormalities causing damage to peribronchiolar attachments and inadequate lung tissue recoil, resulting in increased uncoupling of airways. In asthma, distal airway inflammation and remodelling may also cause airway–parenchyma uncoupling, by altering the mechanical interdependence between these two compartments [8,51]. Similarly to what is described in emphysema, the observed decrease in decorin and lumican in the outer area of small airways in fatal asthma could help to explain structural alterations that have been previously reported at this level, which may lead to disruption of alveolar attachments [8]. The airway–parenchyma uncoupling could explain some of the functional changes observed in asthmatics such as the loss of the deep breath bronchoconstrictor effect in spontaneous bronchoconstrictive episodes, enhanced airway closure, and reduced elastic recoil [8,23].

In conclusion, our results show that PGs are differentially altered in the airways of patients with fatal asthma. The main differences in PG content between fatal asthma and controls were observed in the small
airways, showing a decrease in decorin and lumican content and an increase in versican content. These findings may have implications for our understanding of remodelling in the small airways in fatal asthma.

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