

Mechanical strain enhances proteoglycan message in fibroblasts from asthmatic subjects

M. S. Ludwig*, N. Ftouhi-Paquin*, W. Huang*, N. Pagé†, J. Chakir† and Q. Hamid*

*Meakins Christie Laboratories, McGill University Hospital Center, Montreal, Quebec and †Centre de Recherche, Hôpital Laval – Institute Universitaire de Cardiologie et de Pneumologie, Sainte-Foy, Quebec, Canada

Summary

Background Remodelling of the asthmatic airway includes increased deposition of proteoglycan (PG) molecules. One of the stimuli driving airway remodelling may be excessive mechanical stimulation.

Objective We hypothesized that fibroblasts from asthmatic patients would respond to excessive mechanical strain with up-regulation of message for PGs.

Methods We obtained fibroblasts from asthmatic patients (AF) and normal volunteers (NF) using endobronchial biopsy. Cells were maintained in culture until the fifth passage and then grown on a flexible collagen-coated membrane. Using the Flexercell device, cells were then subjected to cyclic stretch at 30% amplitude at 1 Hz for 24 h. Control cells were unstrained. Total RNA was extracted from the cell layer and quantitative RT-PCR performed for decorin, lumican and versican mRNA.

Results In unstrained cells, the expression of decorin mRNA was greater in AF than NF. With strain, NF showed increased expression of versican mRNA and AF showed increased expression of versican and decorin mRNA. The relative increase in versican mRNA expression with strain was greater in AF than NF.

Conclusions These data support the hypothesis that proteoglycan message is increased in asthmatic fibroblasts subject to mechanical strain. This finding has implications for the mechanisms governing airway wall remodelling in asthma.

Keywords airway remodelling, decorin, lumican, versican

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Introduction

It is well established that the airway wall is remodelled in asthmatic patients [1, 2]. In endobronchial biopsies of asthmatic patients, changes in the reticular basement membrane and the subepithelial layer of the airway wall have been described by a number of investigators [3–5]. Alterations in specific collagen subtypes and in the glycoproteins, tenascin and fibronectin, have been shown, as have increases in various types of proteoglycans (PG) [6, 7].

PGs are extracellular matrix (ECM) molecules which subserve a number of important biologic functions [8]. Because of their hydrophilic structure, they have the capacity to attract water into the ECM, thereby altering tissue turgor and viscoelastic properties of the matrix. They interact with various cytokines and growth factors and affect cell migration and proliferation. PGs also play a key role in collagen fibrillogenesis. We have published data in mild atopic asthmatics showing increases in the large aggregating PG, versican, and the small leucine-rich PGs, lumican and biglycan, in the

subepithelial layer of asthmatic airways [7]. Moreover, increases in the amount of PG were correlated with bronchial hyper-responsiveness. Redington and co-workers [6] have shown increases in decorin, another small leucine-rich PG, in the asthmatic airway wall, while Roberts [9] has reported an abundance of versican and decorin in autopsy specimens of airway from patients who died of fatal asthma or in whom asthma was a contributing factor.

Remodelling of the airway wall is linked to inflammation [1]. Another potential stimulus to remodelling which has received attention of late, is that of mechanical stimulation. Asthmatic airway walls are likely subject to increased mechanical stimulation because of increases in baseline tone of the airways due to bronchoconstriction and because of the excessive transmural pressures generated during normal ventilation. This would result in airway structural cells being exposed to higher stresses than normal. In addition, because of the heterogeneous nature of bronchoconstriction in asthma, airflow and volume are not evenly distributed throughout the lung [10]. Hence, some airways would be subject to relatively greater changes in volume, and the airway structural cells would undergo greater mechanical strain. Along these lines, recent studies by Drazen and co-workers [11–13] have demonstrated that airway epithelial cells submitted to

Correspondence: Dr Mara S. Ludwig, Meakins Christie Laboratories, 3626 St Urbain Street, Montreal, Quebec, Canada H2X 2P2.

E-mail: mara.ludwig@mcgill.ca

increased pressure gradients up-regulate production of message for molecules involved in fibrotic responses, such as transforming growth factor (TGF)- β and endothelin-1.

While relatively little is known about the effects of mechanical stimulation on PG production in the lung, data are available in other organ systems. Cyclic compression of tendon and cartilage leads to up-regulation of PG production [14, 15]. Excessive mechanical strain of lung fibroblast cultures results in increase in other matrix components, e.g. type I collagen [16]. In preliminary experiments, we have shown that mechanical strain results in up-regulation of versican message and protein production in adult human lung fibroblasts [17, 18]. Therefore, we questioned whether excessive mechanical stimulation of asthmatic airway cells would result in enhanced message for PG synthesis, as compared with airway cells from normal controls. To test this hypothesis, fibroblasts were obtained from endobronchial biopsies of asthmatic patients and normal controls. Cells were maintained in culture, then subjected to excessive mechanical strain and the message for the PGs, versican, decorin and lumican measured. We chose these particular PGs as we wanted to sample members of different subclasses of PGs – versican is a large aggregating PG, likely to influence viscoelastic properties of tissues [8], decorin is a class 1, small leucine-rich PG which is known to be up-regulated in asthma and which interacts with TGF- β [6], and lumican is a class 2, small leucine-rich PG which we have previously shown to be increased in the asthmatic airway wall [7].

Methods

Fibroblast cell lines

Primary bronchial fibroblasts were obtained from four asthmatic patients and four healthy volunteers (Table 1). Asthmatic patients had mild disease as characterized by the use of β -agonists only, and normal forced expiratory volume in 1 s (FEV₁). None had ever used inhaled or systemic corticosteroids. Patients had PC₂₀ methacholine (provocative concentration of methacholine to cause a fall in FEV₁ of 20 %) ranging from 0.28–4.21 mg/mL. All asthmatic patients were atopic. All normal subjects were non-atopic and had PC₂₀ methacholine greater than 16 mg/mL. Details on bronchoscopy, endoscopic biopsy, and isolation and pheno-

typing of fibroblasts have been described in previous publications [19, 20]. Briefly, fibroblasts were isolated from bronchial biopsies [19] and characterized by immunofluorescence and flow cytometry using anti-vimentin, and a mouse anti-human fibroblast antigen Ab-1 antibody (Calbiochem, San Diego, CA, USA) that shows no cross-reaction with epithelial cells, endothelial cells, or smooth muscle cells. This identification was used to confirm the purity of our bronchial cell culture [21].

Cell culture

Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco BRL, Life Technologies, NY, USA), streptomycin (100 mg/mL) and amphotericin B (250 ng/mL). Subculturing was carried out using trypsin (0.25%)

Mechanical stimulation of cultured fibroblasts

For all experiments, cells were used at the fifth passage. Cells were subcultured in six-well collagen I-coated flexible bottom plates 24 h prior to the experiment at 2×10^5 cells/well. Prior to initiating stretch, the medium was replaced by DMEM supplemented with 0.5 % fetal bovine serum, streptomycin and amphotericin. Culture plates were placed on a multiport vacuum manifold controlled by a solenoid valve and computer software (FX-3000, Flexercell McKeesport, PA, USA). Application of a negative pressure causes a downward deformation of the flexible silastic membrane to which the cells were attached. A biaxial strain of 30% amplitude was applied for 24 h at a frequency of 1 Hz. In each experiment, duplicate plates were either subjected to cyclic stretch using the Flexercell apparatus or maintained unstretched as a control.

RNA extraction and RT quantitative PCR

Total RNA was extracted from the cell layer using TRizol reagent (Life Technology, Burlington, Canada). The isolated RNA was dissolved in diethylpyrocarbonate-treated water. The concentration of RNA was spectrophotometrically determined. For each experiment, 2 μ g of total RNA was reverse transcribed to cDNA using 100 ng of random primers and Moloney Murine Leukemia Virus (M-MLV) RT as per the manufacturer's protocol. Amplification reactions were performed using 4 μ L from each cDNA reaction with the following reagents: 100 pmole of Hdec2 5'-AGTTAGGT TTCCAGTATCTA-3' and Hdec3 5'-ATGGCTCTCTGGC CAACACG-3' for decorin RT-PCR; 100 pmole of Hlum2 5'-AGGTATTCGAGTCATTTAAG-3' and Hlum 1 (Sens Primer) 5'-GCATCTGCTTTAAGAATTAAC-3' for lumican RT-PCR; 100 pmole of Hvers2 5'-AGGACTTTGGCT GAAATGAT-3' and Hvers1 5'-ACAGACTTCTGAGAGG CCA-3', 200 mM dNTP, 2.5 U of platinum DNA polymerase enzyme and 1 $\times$ platinum DNA polymerase buffer (Gibco BRL). The reactions were carried out in a DNA thermal cycler (MJ Research, Watertown, MA, USA) for 15 cycles for decorin, 25 cycles for lumican and 30 cycles for versican. The number of cycles for each molecule was determined in preliminary experiments in which the linear portion of the saturation curves was defined.

Table 1. Characteristics of subjects from whom bronchial fibroblasts were obtained

Group	Age	Sex	PC ₂₀	FEV ₁ (%)	Atopy	Medication
Asthmatic	24	M	2.33	4.0 (98)	Yes	β -agonist only
Asthmatic	20	F	4.21	3.5 (105)	Yes	β -agonist only
Asthmatic	20	M	0.28	4.2 (98)	Yes	β -agonist only
Asthmatic	20	M	2	4.2 (109)	Yes	β -agonist only
Normal	50	M	256	3.8 (98)	No	No
Normal	38	F	205	3.3 (100)	No	No
Normal	32	M	256	4.0 (119)	No	No
Normal	32	M	25.4	4.2 (95)	No	No

PC₂₀, provocative concentration of methacholine to cause a fall in FEV₁ of 20 %; FEV₁, forced expiratory volume in 1 s; M, male; F, female; % predicted is shown in parentheses.

For measurement of 18S rRNA, 1 µg RNA was reverse transcribed with random primers (Invitrogen, Burlington, Canada). Amplification was carried out with 2 µL of cDNA incubated with 18S primers (5'-CGGCTACCACATCCAG GAAG-3' and 5'-GCTGGAATTACCGCGGCTGC-3') and Taq DNA Polymerase (Invitrogen). The reactions were carried out for 20 cycles in a DNA thermal cycler. The number of cycles to achieve linearity was determined in preliminary experiments.

PCR products were electrophoresed in 1.2% agarose gel containing ethidium bromide. The gels were photographed and the integrated density values were analysed on a FluorChem 8000 imaging System (Alpha Innotech, San Leandro, CA, USA).

Data analysis

The data were analysed using GraphPad software. Data are reported as mean $\pm$ standard error. ANOVA with Bonferroni correction was used to analyse differences among groups. Significance levels were determined at $P < 0.05$. In order to test whether the degree of up-regulation of versican message was greater in asthmatic fibroblast-strain (AF-S) vs. normal fibroblast-strain (NF-S), unpaired *t*-test was applied.

Results

Results of RT-PCR analysis for decorin, lumican and versican are shown in Figs 1–3. The top panel shows agarose gels of PCR products for all of the samples studied, while the bottom figure shows results of quantitative analysis. Asthmatic fibroblast-no strain (AF-NS) showed an increase in message for decorin as compared with normal fibroblast-no strain (NF-NS) ($P < 0.05$). In NF, strain caused an increase in message for versican as compared with NF not subject to mechanical strain ($P < 0.001$). In AF, strain caused an increase in message for decorin and versican ($P < 0.001$). Lumican message was unchanged. When we compared the amount of up-regulation for versican mRNA in response to mechanical stimulation in AF vs. NF, we found that the

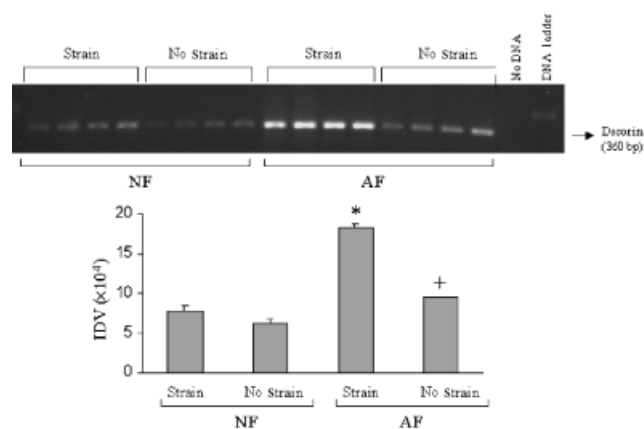


Fig. 1. Decorin mRNA in normal fibroblasts (NFs) and asthmatic fibroblasts (AFs) submitted to strain or control conditions. The top panel shows the agarose gel of the PCR product for all samples and the bottom panel shows the quantitation of the gel. + $P < 0.05$ vs. NF, no strain; * $P < 0.001$ vs. AF, no strain.

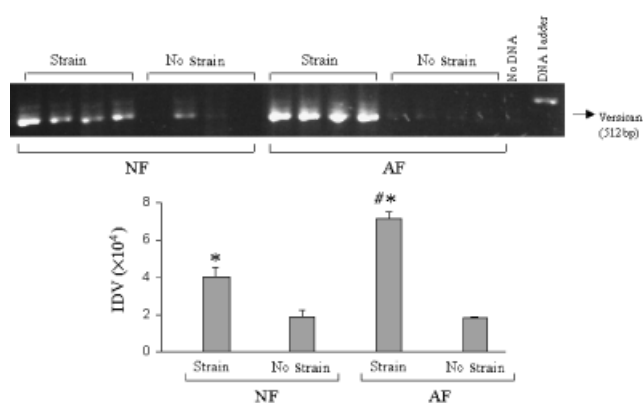


Fig. 2. Versican mRNA in normal fibroblasts (NFs) and asthmatic fibroblasts (AFs) submitted to strain or control conditions. The top panel shows the agarose gel of the PCR product for all samples and the bottom panel shows the quantitation of the gel. * $P < 0.001$ vs. AF, no strain, and NF, no strain, respectively. The relative increase in versican mRNA after strain was greater in AF vs. NF * $P < 0.01$.

increase in message was significantly greater in AF ($P < 0.01$). (We do not report all the significant comparisons among the data, e.g. NF-S was different from AF-S, only those we felt were of greatest physiologic interest.)

Saturation curves were defined for each PCR product. The slope of PCR product vs. cycle number was linear for decorin at 15 cycles, for lumican at 25 cycles and for versican at 30 cycles.

Results for the housekeeping gene, 18S, are shown in Fig. 4. While the message for 18S showed modest variation among groups, the degree of variation was not sufficient to explain the PG results. PG message was not corrected for 18S message.

Discussion

The results of this study provide the first evidence that fibroblasts from asthmatic patients as compared with fibroblasts from normal volunteers, are differentially affected by mechanical strain. Message for the PGs, decorin and

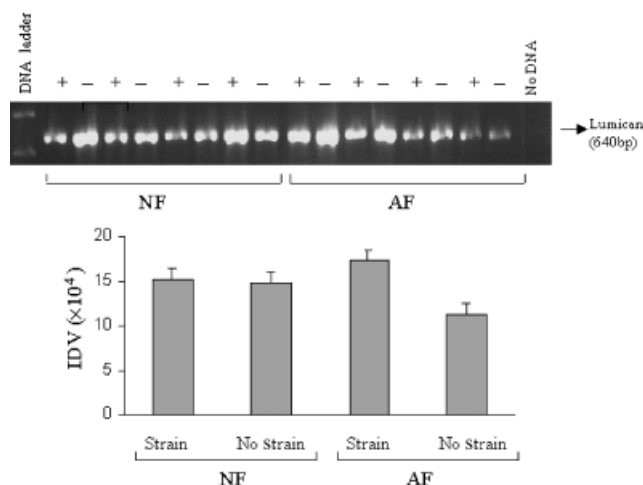


Fig. 3. Lumican mRNA in normal fibroblasts (NFs) and asthmatic fibroblasts (AFs) submitted to strain or control conditions. The top panel shows the agarose gel of the PCR product for all samples and the bottom panel shows the quantitation of the gel. +, strain; -, no strain.

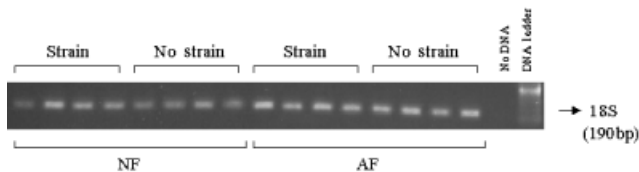


Fig. 4. 18S rRNA in samples from normal fibroblasts (NFs) and asthmatic fibroblasts (AFs).

versican, ECM molecules important in the process of airway wall remodelling, was significantly increased in response to a mechanical stimulus. These data have important implications for understanding the mechanisms underlying airway wall remodelling in asthma.

There is ample evidence that the airway wall is remodelled in asthma [3–5]. Recently, attention has been directed at alterations in PGs in the airway wall. We have shown in endobronchial biopsies taken from asthmatic patients that PGs are increased in the submucosal layer as compared with biopsies from normal volunteers; moreover, the increase was correlated with airway hyper-responsiveness [7]. Redington and co-workers have shown, also in endobronchial biopsy specimens, that decorin, a small leucine-rich PG which interacts with TGF- β and which is important in collagen fibrillogenesis, is increased in the subepithelial connective tissue. Finally, Roberts [9] has analysed autopsy specimens from patients dying of fatal asthma and shown prominent deposition of several classes of PGs both in the submucosa and around smooth muscle cells.

There is also some information available in *in vitro* systems. Airway smooth muscle passively sensitized with serum from an asthmatic patient, showed increased production of versican, decorin and chondroitin sulphate, as compared with smooth muscle exposed to serum from a non-asthmatic individual [22]. Westergren-Thorsson et al. [23] have examined PG production by the same fibroblast populations as were studied in the current experiment. They documented increased production of perlecan, versican, biglycan and small heparan sulphate PG. Moreover, increases in these molecules correlated with airway responsiveness. Of note, decorin production was neither up-regulated in these cells nor correlated with methacholine responsiveness. In our study, decorin message was the one signal increased in the asthmatic, non-strained cells as compared with non-asthmatic, non-strained cells. Further, the linear portion of the PCR product vs. cycle number curve was attained at the fewest number of cycles for decorin, as compared with the other PG molecules. This suggests that post-transcriptional regulation is important in determining decorin secretion in these cells. Alternately, decorin is known to bind to TGF- β and render this molecule relatively unavailable [24]. Insofar as fibroblasts can actively secrete TGF- β [25], it is possible that an inability to measure an increase in decorin in the former study may be related to this mechanism. Lumican and versican mRNA in non-strained, normal cells was not increased as compared with non-strained, asthmatic cells, despite evidence from previous studies showing that these proteins are increased in the asthmatic airway wall [7, 9]. One potential explanation is that cell types other than fibroblasts, e.g. smooth muscle cells, may be involved in the excess production and secretion of these molecules. Inflammatory mediators present in the asthmatic

airway wall may also be required for up-regulation of these molecules.

Inflammation as a mechanism driving airway wall remodelling has been a source of much attention. Data have been published which both support and question this association [26–28]. Recently, Tschumperlain and Drazen [29] have suggested the hypothesis that mechanical stimulation may be important in giving rise to structural changes in the airway wall. They argue that the asthmatic airway is subject to excessive mechanical stress because of airway smooth muscle constriction and alterations in the geometry of the airway lumen. Along these lines, they have shown in cultured airway epithelial cells, that increased transmembrane pressure results in enhanced signal for growth factors known to stimulate fibrosis, such as TGF- β and endothelin-1 [11]. Moreover, exposure of fibroblasts to media from mechanically stimulated bronchial epithelial cells resulted in increased synthesis of fibrous protein as assessed by hydroxyproline incorporation [13]. Data from our laboratory have reported on the effect of excessive mechanical stretch on ECM protein production by fibroblasts [17, 18]. Both versican message and protein production were up-regulated by excessive stretch of fibroblast cell monolayers. A similar result has been reported for type I collagen [16]. In the current study, fibroblasts from healthy volunteers showed increased message for versican with mechanical stimulation, as had been shown in our previous work.

Asthmatic fibroblasts, however, showed a greater sensitivity to mechanical stimulation than did fibroblasts from healthy volunteers. Versican and decorin showed significant increases in mRNA after 24 h of mechanical stretch. The relative increase in versican in AF was greater than that observed in NF. The enhanced sensitivity of AFs to this stimulus makes the mechanism of mechanical stimulation in contributing to airway wall remodelling potentially more important than had previously been appreciated. The importance of this mechanism may also account for the difficulties in explaining remodelling solely as a consequence of inflammation.

The functional implications of increased PG secretion in the airway wall are several. PGs are molecules which have the capacity to affect tissue viscoelastic properties because of their ability to alter tissue turgor [8]. We have shown that digestion of glycosaminoglycan side-chains of PG molecules affects mechanical characteristics of isolated parenchymal strips [30]. Altered deposition of PG by airway fibroblasts could serve as an adaptive mechanism to excessive mechanical strain – changes in the viscoelastic properties of the matrix could modulate the transmission of mechanical stress to the structural airway cells, and thereby protect against mechanical injury. It is interesting to note that versican, the large PG which forms aggregates with hyaluronic acid and which, therefore, is most likely to affect tissue viscoelastic properties, was the one PG increased in both NF and AF subject to strain. Moreover, the relative increase in versican mRNA was greater in AF, suggesting an up-regulation of this potentially protective mechanism in the asthmatic airway wall.

Altered PG metabolism could also be critical because of the documented role of decorin, as well as other PG molecules, in collagen fibrillogenesis. We have recently shown that the lungs of decorin-deficient mice have decreased airway resistance and increased lung compliance, possibly on the

basis of altered collagen fibril synthesis [31]. The impact of changes in airway mechanical properties and parenchymal recoil on airway hyper-responsiveness is well described [32, 33]. Finally, up-regulation of decorin may be important because of interactions between decorin and TGF- β [24]. TGF- β is clearly important in the remodelling process of the asthmatic airway wall [26, 34]; the presence of decorin could modulate its activity.

In conclusion, we have shown that mechanical stimulation up-regulates message for the PG molecules, decorin and versican. These molecules contribute importantly to the structural and functional characteristics of the airway wall. Mechanical strain as an active stimulus to airway wall remodelling must be considered as part of the paradigm. Further experiments to document potential interactions between the two stimuli to airway wall remodelling, i.e. inflammation and increased mechanical strain, will be especially pertinent.

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