



Differential effects of extracellular matrix and mechanical strain on airway smooth muscle cells from ovalbumin- vs saline-challenged Brown Norway rats[☆]

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ABSTRACT

The asthmatic airway is characterized by alterations in decorin and biglycan and increased airway smooth muscle (ASM). Further, the asthmatic airway may be subjected to abnormal mechanical strain. We hypothesized that ASM cells obtained from ovalbumin (OVA) – and saline (SAL) – challenged rats would respond differently to matrix and mechanical strain. ASM were seeded on plastic, decorin or biglycan. Additional cells were grown on decorin, biglycan or collagen type 1, and then subjected to mechanical strain (Flexercell). The number of OVA ASM was significantly greater than SAL ASM when seeded on plastic. A significant decrease was observed for both OVA and SAL ASM seeded on decorin compared to plastic; the reduction in ASM number was more modest for OVA. Biglycan decreased SAL ASM number only. Strain reduced cell number for SAL and OVA ASM grown on all matrices. Strain affected expression of β_1 -integrin differently in OVA vs SAL ASM. These data suggest that matrix and mechanical strain modulate ASM number; these effects are differentially observed in OVA ASM.

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1. Introduction

Asthma is a disease characterized by inflammation, reversible airway obstruction and structural alterations termed airway remodeling (Vignola et al., 2000). The structural changes include an increased deposition of extracellular matrix (ECM) proteins within and around the airway smooth muscle (ASM) in addition to an increase in the ASM mass (Black et al., 2003). This increase has been shown to be due to both hyperplasia and hypertrophy (Ebina et al., 1993) and may occur through increased cell division or decreased apoptosis (Hirst et al., 2004). Deposition of collagen and fibronectin in the subepithelial basement membrane of asthmatic airways (Roche et al., 1989) has also been well established. Recent data have shown that proteoglycans (PGs) contribute to airway remodeling. PGs are macromolecules consisting of a protein core and glycosaminoglycan (GAG) side chains (Pini et al., 2007; Redington et al., 1998; Shimizu-Hirota et al., 2004). Of special interest are the small leucine rich PG subclass, including decorin and biglycan, which show very high homology (Iozzo, 1998) and which have significant effects on a variety of cellular functions relevant to asthma. Decorin is evident in both human and rat airways, mainly

in the subepithelial connective tissue and, more so, the adventitial layer (Redington et al., 1998; Pini et al., 2006). Biglycan, on the other hand, is found mainly within and around ASM bundles and in the subepithelial region (Pini et al., 2006; Huang et al., 1999).

PGs have been shown to serve a number of biologically important roles, affecting collagen fibrillogenesis and matrix assembly (Iozzo, 1998), binding to different growth factors (Hildebrand et al., 1994) and altering lung mechanics (Fust et al., 2005). The role of decorin and biglycan in the control of cell growth has also been studied. While evidence has shown that biglycan enhances proliferation and migration of vascular smooth muscle cells (Shimizu-Hirota et al., 2004), decorin may have anti-proliferative and/or pro-apoptotic effects, depending on the cell type studied. D'Antoni et al. (2008) showed that growth of non-asthmatic human ASM cells on various ECM components resulted in differential regulation of cell growth. Decorin induced a reduction in cell number that was primarily due to an increase in apoptotic rates; decrease in proliferation was more modest. On the other hand, collagen increased ASM cell number.

Another potential contributor to asthma airway pathology is abnormal mechanical strain (Elias, 2000). The structural cells of the airways, which include epithelial cells, fibroblasts and ASM cells, are capable of sensing and responding to their mechanical environment (Tschumperlin and Drazen, 2001). It is likely that, as a result of airway remodeling and heterogeneous airway constriction, some airways may be subjected to abnormal volume alterations, which result in transmission of greater mechanical strain to airway

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structural cells (Lyons and McFadden, 1967). Different factors involved in the mechanoresponsiveness of cells include stretch-activated ion channels, mechanoreceptors and matrix-integrin-cell interactions (for review see Liu et al., 1999). The last provides a direct route for mechanical signals to be transmitted into the cell. Vascular smooth muscle cells subjected to mechanical stretch showed increases in both expression of β_1 -integrin and rates of apoptosis (Wernig et al., 2003). Subsequent experiments revealed that functional blocking of the β_1 -integrin inhibited this response, thus suggesting a role for integrins in mediating stretch-induced apoptosis. Bonacci et al. (2003) have shown a decrease in cell number in response to strain in bovine ASM cells grown on collagen and laminin matrices. It is possible that ASM cells from an asthmatic animal model may show enhanced sensitivity to the effects of mechanical strain, due to pre-conditioning of the cells to mechanical stimulation.

In order to investigate the effects of abnormal mechanical stimulation and altered extracellular matrix on ASM proliferation, ASM cells were obtained from the airways of Brown Norway (BN) rats sensitized with ovalbumin (OVA) and either challenged with OVA or saline (SAL). ASM cells were cultured on plastic, decorin and biglycan and cell number was determined. In a second set of experiments, cells were cultured on collagen, decorin and biglycan and subjected to mechanical strain; the effects on cell number and integrin expression were determined.

2. Materials and methods

The following reagents were purchased from Gibco-BRL-Invitrogen (Burlington, Ontario, Canada): Dulbecco's modified Eagle medium (DMEM), F-12 Nutrient Mix (HAM), antibiotic-antimycotic, fetal bovine serum (FBS), trypsin-EDTA solution and Hanks' balanced salt solution (HBSS). Decorin, biglycan, collagen Type 1, transferrin, insulin, ascorbic acid, bovine serum albumin (fraction V) (BSA), monoclonal anti- α smooth muscle actin, anti-calponin, anti-pan cytokeratin, ovalbumin and tetramethylethylenediamine (TEMED) were purchased from Sigma (Oakville, Ontario, Canada). To detect antibodies, Alexa Fluor 568 signal amplification kit was purchased from Molecular Probes (Burlington, Ontario, Canada). PDGF was purchased from R&D Systems (Minneapolis, MN, USA). β_1 -Integrin antibody (cat# sc-6622) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Molecular markers, HRP Chemiluminescence Kit and precision streptactin HRP were purchased from Bio-Rad laboratories (Hercules, CA, USA). BCA Protein Assay Kit was purchased from Pierce (IL, USA).

2.1. Airway smooth muscle cell isolation

2.1.1. Brown Norway rat model of allergic asthma

Cells used in *in vitro* studies were obtained from the BN rat model of allergic asthma as previously described (Pini et al., 2006). To induce allergen-driven experimental asthma, allergic sensitization was performed on 7–8-week-old BN rats by a single subcutaneous injection of 1 mg of OVA with 100 μ g aluminum hydroxide adjuvant in phosphate buffered saline (PBS) solution. Heat-killed *Bordetella pertussis* (2×10^9 bacilli) was given intraperitoneally (ip). Two weeks later, aerosolized OVA or SAL challenges were delivered through orotracheal intubation repeatedly on days 14, 19 and 24. On the day of challenge, a 5% (w/v) solution of OVA was prepared in sterile saline. Xylazine (7 mg/kg ip) was used to sedate the rats, after which the animals were anesthetized with sodium pentobarbital (30 mg/kg ip). The polyethylene tubing used to intubate the rats was inserted into a Plexiglas box into which a micromist nebulizer (Hudson RCI; Teleflex Medical, Temecula, CA,

USA) delivered aerosols of OVA or SAL for 5 min at a rate of 8 l/min. Forty-eight hours following the last antigen challenge, animals were sacrificed with 1 ml injection (ip) of sodium pentobarbital, and smooth muscle cells isolated from the trachea. All experimental studies conducted on animals were performed using protocols approved by McGill University Animal Care Committee in compliance with the Guide to the Care and Use of Experimental Animals devised by the Canadian Council of Animal Care.

2.1.2. Cell isolation and cell culture

Tracheas were removed from both groups under aseptic conditions, incubated in an enzyme solution containing HBSS with 0.006 g of collagenase type IV (0.2% of 3 ml HBSS) and 0.0015 g of elastase type IV (0.05% of 3 ml HBSS) and then placed in a water bath. These enzymes were specifically chosen due to the high content of collagen and elastin in the matrix surrounding ASM (Hirst, 1996). Following digestion, the connective tissue was filtered out and the remaining cell suspension was centrifuged in order to isolate ASM cells. The cell pellet was re-suspended in 10% FBS in DMEM (supplemented with L-glutamine, 110 mg/ml sodium pyruvate, and pyroxidine hydrochloride), F-12 medium (supplemented with L-glutamine) containing an antibiotic-antimycotic cocktail and plated in a 25 cm² culture flask. The medium was changed every 48 h. For all experiments, passages 1–5 were used.

2.1.3. Indirect immunofluorescence: smooth muscle-specific proteins

The identity of rat ASM cells was confirmed by positive immunocytochemical staining for smooth muscle specific- α actin and calponin (data not shown). These proteins were chosen as they have been shown to be specific to ASM and are not present in fibroblasts and epithelial cells (two cell types which might contaminate cell cultures upon isolation of smooth muscle cells) (Halayko et al., 1996). Contamination of cultures by epithelial cells was also examined by negative staining for keratin. Cells were grown to post-confluence on glass chamber wells in the presence of DMEM-F12 medium containing 0.5% FBS, 0.4 μ g/ml insulin, 5 μ g/ml transferrin, 100 μ M ascorbic acid, 0.1% BSA and antibiotic-antimycotic cocktail (0.5% FBS medium). Cells were serum starved in order to maximize expression of contractile proteins (Halayko et al., 1996). Medium was removed and glass chambers containing cells were gently washed with PBS solution. In order to permeabilize cells, 300 μ l of ice-cold acetone (-20°C) was added to each chamber well and kept for 20 min at -20°C , after which, acetone was removed and replaced with 70% ice-cold ethanol for 5 min, also at -20°C . The following steps were performed at room temperature. The chamber wells were removed leaving only a glass slide to which the cells had been fixed. Slides were rinsed 3 times with PBS for 5 min/rinse in order to rehydrate cells. The slides were then immersed in 1% BSA solution for 30 min in order to block any unspecific binding sites. The previous washing step was then repeated. Primary antibodies for α -smooth muscle actin, calponin and keratin were added (diluted 1:400, 1:10,000 and 1:400 in PBS solution, respectively). Slides were incubated in a humidified plastic container for 1 h. Following the incubation period, slides were washed, blot-dried and the secondary antibody (10 μ g/ml recommended concentration for rabbit anti-mouse IgG) was applied for 30 min followed by a 30 min incubation period with 10 μ g/ml goat anti-rabbit IgG in order to enhance the fluorescence signal. Positive cells were visualized using Image Pro-Plus software.

2.1.4. Surface coating of culture well with extracellular matrix

Decorin and biglycan, purified from bovine articular cartilage, were diluted in PBS to concentrations of 0.1, 0.3 and 1.0 μ g/ml. The diluted proteins were adsorbed to six well plastic culture plates and incubated over night at 37°C . Plates were then washed with

PBS to remove unbound proteins followed by a 30 min incubation period at room temperature with 0.1% BSA in PBS to block any unoccupied binding sites (D'Antoni et al., 2008; Hirst et al., 2000). [In preliminary experiments, we coated the wells with concentrations of matrix similar to those used previously in human ASM cells (D'Antoni et al., 2008).] The rat ASM seemed to be much more sensitive to the anti-proliferative effects of decorin and biglycan; hence, we reduced the concentrations of these proteins, so that cell growth would not be completely impeded at lesser concentrations.

2.2. Culture of ASM cells on different matrices

2.2.1. Cell culture protocol

Cells were seeded onto 6-well culture plates at a density of 4.1×10^2 cells/cm² in the presence of 0.5% FBS medium and allowed a growth arrest period of 48 h. Following serum starvation, cells were stimulated with 10 ng/ml of the mitogen PDGF in 0.5% FBS medium (0 h time point) and medium was replenished every 48 h thereafter. Cells were grown on plastic or on varying concentrations of matrix-coated wells as described above. The baseline control consisted of cells seeded on plastic in the presence of 0.5% FBS medium. As a positive control, cells were seeded on plastic in 10% FBS medium. Cells in all culture conditions were stimulated with PDGF at 0 and 48 h, except for the positive control. At 0, 48 and 96 h ASM cells were harvested by washing twice with PBS to remove medium and adding trypsin-EDTA solution to detach cells. Neutralization of trypsin was achieved via the addition of 1:5 (v/v) of 10% FBS medium. Following centrifugation (5 min, 1500 rpm), cells were re-suspended in 0.5% FBS medium and counted using the trypan blue viability test and a haemocytometer chamber.

2.2.2. Mechanical stimulation of airway smooth muscle cells

2.2.2.1. Cell culture protocol on silastic culture plates. Six-well culture plates (BioFlex-untreated plates, Flexcell International Corporation, Hillsborough, NC, USA) composed of a flexible silastic bottom membrane were coated in our laboratory with biglycan, decorin (both 0.1 µg/ml) and collagen (rat tail, 1.0 µg/ml) as described above. While growth on plastic served as a control in the first set of experiments, for mechanical stretching of cells, collagen coating was necessary as a control (to compare the decorin and biglycan results) because cells grew poorly on the silastic membrane. Cells were seeded onto culture plates in the presence of 10% FBS medium for 48 h in order to allow for appropriate attachment and spreading onto the silastic membrane. Cells remained in 0.5% FBS medium for 24 h prior to strain initiation, after which cells were mechanically strained for 48 h in the presence of 10% FBS medium.

2.2.3. Mechanical strain of airway smooth muscle cells

The Flexercell Strain Unit (FX-3000, Flexercell McKeesport, PA, USA) was used in order to mechanically strain cells. Following a 24 h period of serum starvation (0 h time point) culture plates were placed onto a vacuum manifold controlled by computer software and a solenoid valve. The system uses a vacuum source to apply a negative pressure causing a downward deformation of the membrane to which the cells are attached. The strain applied over the loading-post regions is approximately equal in the radial and circumferential directions (Van de Geest et al., 2004). At strain initiation, cells were replenished with 10% FBS medium and subjected to an equibiaxial strain of 17% amplitude for 48 h at a frequency of 0.2 Hz. This strain regimen was chosen in order to apply an excessive strain on the cells. Previous research has shown that if muscle stretches isotropically as the cube root of lung volume, then a 17% strain would appear to be within the physiological range for airway distension during deep inspiration (Raboudi et al., 1998). A frequency of 0.2 Hz was specifically chosen to allow for the maximum amount of strain possible; that is, an inverse relationship

exists between frequency and strain. Cell number was assessed 48 h following mechanical strain using the trypan blue exclusion test and haemocytometer chamber.

2.3. Western blot analysis for expression of β_1 -integrin in airway smooth muscle cells

Following mechanical strain cell lysates were obtained by incubating the cells with lysis buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, and 1% Nonidet P-40 (NP-40) in Tris-buffered saline (TBS) containing proteinase inhibitors for 1 h at 4 °C. The cell lysates were then pelleted by centrifugation at $1500 \times g$ for 15 min and supernatants were used to determine the protein content (Bradford, 1976).

Equal amount of protein (25 µg) was loaded and run on a 10% SDS-PAGE gel, after which separated proteins were transferred to a nitrocellulose membrane. In order to prevent non-specific binding, the membrane was incubated with 5% skim milk in Tris buffered saline solution (TBS; 0.02 M Tris; 0.5 M NaCl; pH 7.6) with 0.1% Tween 20 (TBS-T) for 1 h at room temperature. The membranes were then probed with primary antibody against β_1 -integrin (diluted 1:200) overnight at 4 °C. Membranes were then washed with TBS-T and probed with horseradish peroxidase-labelled secondary antibodies (diluted 1:5000) for 1 h at room temperature followed by further washing with TBS-T. Enhanced chemiluminescence was performed to visualize immunoreactive bands. Densitometric analysis of β_1 -integrin was performed using image analyzer software (Fluorchem; Alpha Innotech, San Leandro, CA, USA). Software measures the sum of all pixel values following background correction.

2.4. Statistical analysis

Graphpad software was used to analyze these data and results were expressed as mean $\pm$ SE. Analysis of variance (ANOVA) with Dunnett's multiple comparison test was used to analyze differences in cell number among matrix concentrations compared to baseline control. A two-way ANOVA was used to identify differences between cell groups. An independent *T*-test was performed to verify differences between strain and no strain groups.

3. Results

3.1. Cell number is increased in OVA cells when compared to SAL cells

OVA cell number was significantly increased as compared to SAL cell number at 48 h under baseline conditions (0.5% FBS, plastic) ($P < 0.05$) (Fig. 1). These differences were also apparent when cells were seeded on 0.1 and 1.0 µg/ml decorin matrices ($P < 0.01$ and $P < 0.05$, respectively) and positive control (10% FBS, plastic) ($P < 0.001$).

3.2. Effect of extracellular matrix proteins on airway smooth muscle cell number

3.2.1. Effect of decorin on airway smooth muscle cells

ASM cells were seeded onto 6-well culture plates coated with decorin (0.1, 0.3 and 1.0 µg/ml). Cell number was assessed initially at the point of stimulation with PDGF (0 h) and 48 and 96 h post-stimulation (Fig. 2). At the 0 h time point, cell number did not differ compared to 0.5% FBS, plastic, regardless of cell group or matrix coating. In SAL cells, a significant reduction in cell number was observed compared to 0.5% FBS, plastic for cells seeded on a 1.0 µg/ml decorin at 48 h ($P < 0.05$) (Fig. 2a). Seeding OVA cells on the same concentration of decorin (1.0 µg/ml) significantly

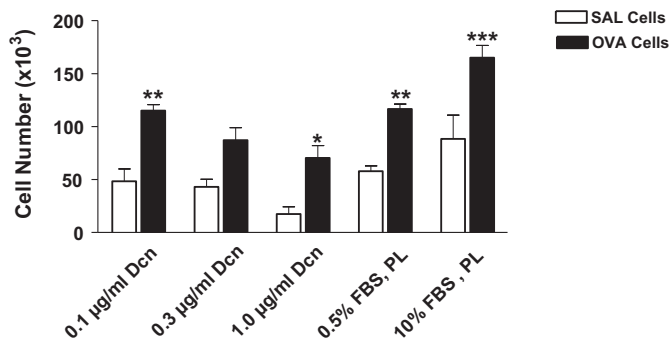


Fig. 1. Comparison of cell number between OVA and SAL cells. Following 48 h of PDGF stimulation, cell number was assessed for OVA and SAL cells grown on plastic (PL) and decorin (Dcn). Data are represented as mean $\pm$ SE ($n=3$). * $P<0.05$ vs SAL cells and ** $P<0.01$ vs SAL cells and *** $P<0.001$ vs SAL cells.

reduced cell number, compared to 0.5% FBS, plastic also at 48 h ($P<0.01$) (Fig. 2b).

3.2.2. Effect of biglycan on airway smooth muscle cells

Seeding SAL cells on a biglycan (1.0 µg/ml) matrix caused a decrease in cell number as compared to 0.5% FBS, plastic at 48 h ($P<0.05$) (Fig. 3a). However, seeding OVA cells on a biglycan matrix did not affect cell number, relative to 0.5% FBS medium, regardless of biglycan concentration and/or time point (Fig. 3b).

3.2.3. OVA cells may be more resistant to the decorin-induced reduction in cell number

Cell number was significantly increased for OVA cells (Fig. 1) and OVA cells also appeared to be more resistant to the decorin-induced reduction in cell number. SAL cell number was markedly diminished when cells were grown on a 1.0 µg/ml decorin matrix,

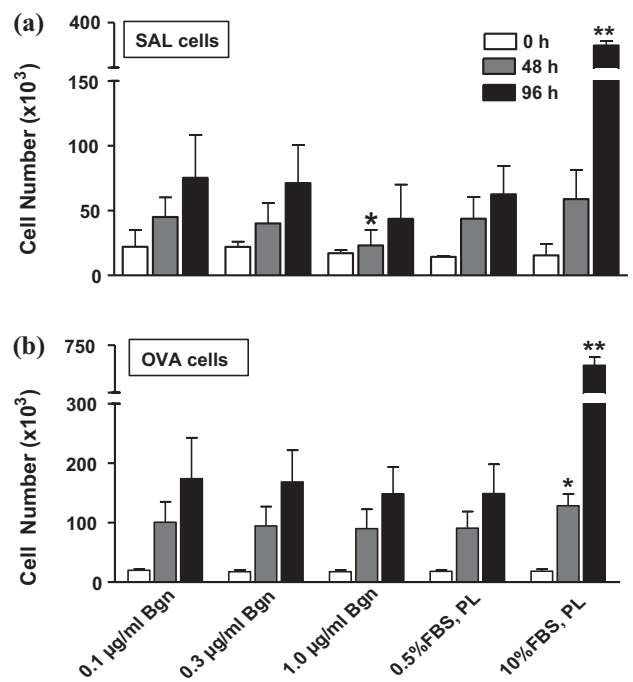


Fig. 3. Effect of biglycan on ASM cell number. SAL cells (a) and OVA cells (b) were seeded on a biglycan (Bgn) matrix and cell number was assessed at 0, 48 and 96 h following PDGF stimulation. Data are represented as mean $\pm$ SE ($n=3$). * $P<0.05$ vs 0.5% FBS, PL and ** $P<0.01$ vs 0.5% FBS, PL at equivalent time point.

showing a 70% decrement in cell number when compared to baseline control (at 48 h). On the other hand, OVA cells showed only a 30% relative reduction in cell number (at 48 h) when grown on the same concentration of decorin. Growth on biglycan caused a 52% reduction in cell number for SAL cells, whereas OVA cells were unaffected by growth on this PG (at 48 h).

3.3. Effect of mechanical strain and extracellular matrix on airway smooth muscle cells

3.3.1. SAL and OVA cells respond to mechanical strain

Both SAL and OVA cells were subjected to an equibiaxial strain of 17% amplitude applied for 48 h at a frequency of 0.20 Hz; control cells were not strained. When compared to no strain (NS), 48 h of mechanical strain (S) resulted in a significant decrease in cell number for SAL cells seeded on decorin, biglycan and collagen ($P<0.05$) (Fig. 4a). OVA cells responded in a similar manner (Fig. 4b); strain resulted in a significant reduction in cell number for cells seeded on all matrices ($P<0.05$).

3.3.2. Decorin may enhance the strain-induced reduction in cell number for OVA cells

We compared the ratio of decrement in cell number (S vs NS) as a result of ECM for SAL and OVA cells. For SAL cells, growth on decorin matrix, as compared to growth on collagen matrix (as a positive control), did not significantly affect the response to mechanical strain (Fig. 4a). On the other hand, growth on decorin matrix resulted in a significant enhancement of the strain-induced reduction in cell number for OVA cells, when compared to the positive control, collagen ($P<0.05$) (Fig. 4b). That is, strain resulted in a 70.2% reduction in cell number for OVA cells grown on decorin, while cells grown on collagen showed only a 47.3% decrement in cell number in response to mechanical strain. There was no differential response to the mechanical strain-induced decrease in cell number for either SAL or OVA cells when cells were seeded on biglycan, relative to collagen.

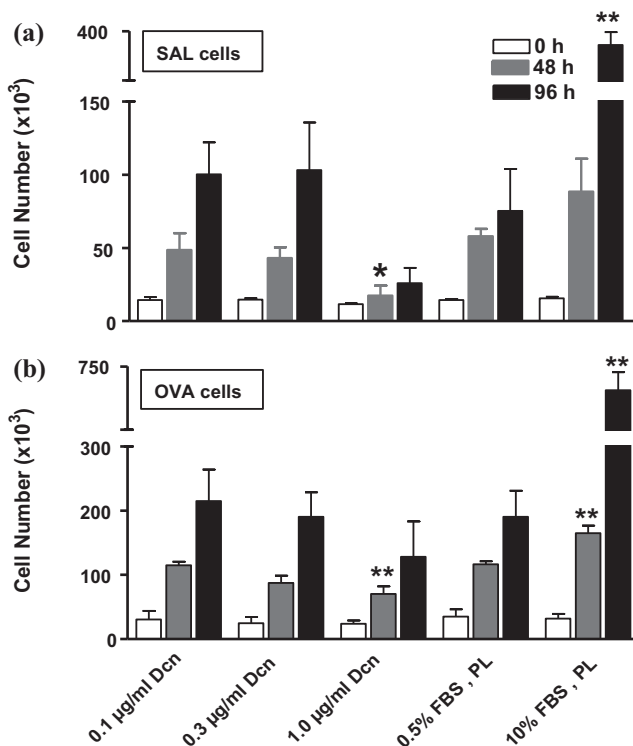


Fig. 2. Effect of decorin on ASM cell number. SAL (a) and OVA cells (b) were seeded on decorin matrix and cell number was assessed at 0, 48 and 96 h following PDGF stimulation. Data are represented as mean $\pm$ SE ($n=3$). * $P<0.05$ vs 0.5% FBS, PL and ** $P<0.01$ vs 0.5% FBS, plastic (0.5% FBS, PL) at equivalent time point.

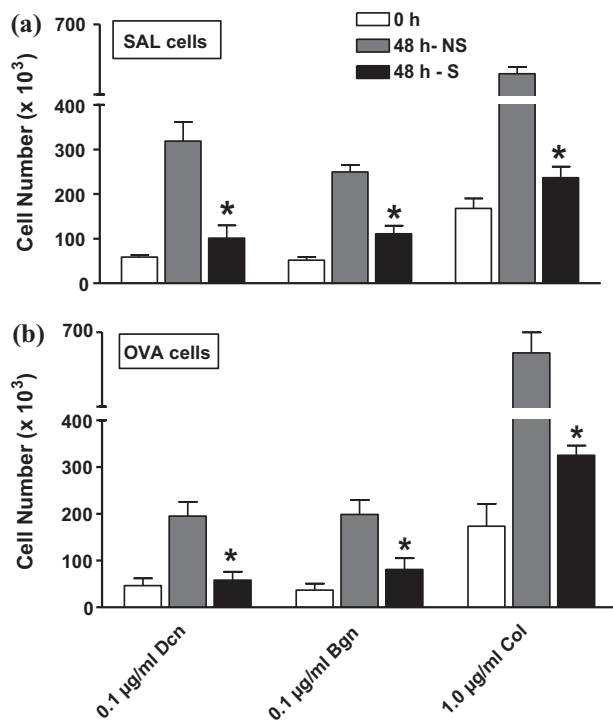


Fig. 4. Effect of mechanical strain and extracellular matrix on ASM cells. Cell number was assessed for SAL cells (a) and OVA cells (b) following 48 h of mechanical strain (S) on decorin, biglycan and collagen (Col). Under all conditions, cell number was decreased by mechanical strain. Data are represented as mean $\pm$ SE ($n = 4$). * $P < 0.05$ vs 48 h no strain (NS) for equivalent matrix.

3.3.3. Effect of mechanical strain on expression of β_1 -integrin in airway smooth muscle cells

SAL cells responded to mechanical strain with a down-regulation of β_1 -integrin expression relative to NS (Fig. 5a). This was observed for cells seeded on all three matrices, decorin, biglycan and collagen. OVA cells up-regulated β_1 -integrin expression in response to mechanical strain, relative to no strain, on all three matrices (Fig. 5b). However, these differences did not attain statistical significance.

4. Discussion

The present study reports the regulation of rat ASM cell proliferation by proteoglycans and mechanical strain. Culture of rat ASM cells on a decorin matrix resulted in a reduction in cell number for both SAL and OVA cells. This effect, however, seemed to be more modest for ASM cells obtained from the BN rat model of allergic asthma. Both SAL and OVA cells responded to mechanical strain with a reduction in cell number; growth on a decorin matrix enhanced this strain-induced reduction in cell number in OVA cells only. Furthermore, expression of the β_1 -integrin subunit was increased in OVA cells and decreased in SAL cells subjected to mechanical stimulation.

These data are consistent with data previously reported in the literature. Various studies have shown an anti-proliferative and/or pro-apoptotic role for decorin in cells of different origin, including carcinoma cell lines (Tralhao et al., 2003), vascular cells (Iozzo et al., 1999; Santra et al., 1997) and, most recently, human ASM cells (D'Antoni et al., 2008). Decorin may serve a potentially protective role in the asthmatic airway as there is evidence showing decorin is deposited in the subepithelial (Pini et al., 2007; Redington et al., 1998) and adventitial layers (Pini et al., 2007) of airways. Decorin could serve to limit enhanced

ASM volume by restricting growth to its usual compartment. Both Pepe et al. (2005) and Benayoun et al. (2003) have reported the encroachment of ASM into the subepithelial region in asthmatic airways. de Medeiros Matsushita et al. (2005) found a significant reduction in decorin in airways of fatal asthmatic subjects compared to controls. It is possible that decreases in decorin deposition could allow unimpeded ASM growth. Furthermore, results from the current study show that OVA cells seemed to be more resistant to the anti-proliferative/pro-apoptotic effects of decorin.

Asthmatic ASM cells may be more proliferative than non-asthmatic ASM cells (Johnson et al., 2004; Triant et al., 2011) and this may contribute to the characteristic increase in ASM in asthmatic airways. Our studies demonstrated that cells obtained from OVA-challenged rats showed an increase in cell number when compared to SAL cells. The literature also reports a phenotypic instability of ASM cells in that they may modulate from a relatively, inactive contractile phenotype to a synthetic, proliferative phenotype (Hirst, 1996; Halayko et al., 1996). It is possible in our experiments that the increase in OVA cell number is due to an enhanced proliferative capacity of the cells. In turn, these cells may become more synthetic, actively synthesizing and secreting growth factors and pro-proliferative matrix components. Johnson et al. (2004) reported that human asthmatic ASM cells increased production of perlecan and collagen I and proliferated faster in response to these matrix proteins, relative to non-asthmatic cells. Hirst et al. (2000) have shown that human ASM cells increased proliferation when grown on collagen and fibronectin matrices. In addition, ASM cells can synthesize and secrete TGF- β , a mitogen capable of inducing ASM proliferation (Xie et al., 2007). OVA cells may be more resilient to the anti-mitogenic/pro-apoptotic effects of decorin because they are more proliferative and thus better able to produce growth factors and matrix proteins that promote cellular growth. As such, the OVA cells examined in the current experiment would be capable of modulating the decorin-induced reduction in cell number. Taken together, these observations suggest that asthmatic ASM cells may be better suited to resist the effect of this matrix protein. A mechanism such as this may result in an enhanced ASM volume and subsequent AHR.

This hypothesis is also supported by results showing that SAL cells seeded on a biglycan matrix decreased in cell number, while OVA cells were unaffected. Little information is available in the literature on the role for biglycan in cell cycle control. One study showed that this proteoglycan enhanced proliferation and migration of vascular smooth muscle cells (Shimizu-Hirota et al., 2004). Conversely, our laboratory has previously shown that biglycan has a modest effect in reducing human airway smooth muscle cell number (D'Antoni et al., 2008). Those experiments showed that, although decorin reduced cell number at all time points, biglycan decreased cell number only at the earliest time point examined; with longer time in culture, cell number had recovered. Again, it is possible that in the present study, OVA cells were more synthetic, thus more robust, and capable of resisting the anti-proliferative or pro-apoptotic signals, biglycan may convey.

One potential factor confounding our experimental results is that the relative number of myofibroblasts may have been increased in the OVA cell cultures. Airway myofibroblasts have been shown to be increased in human asthma (Beguere et al., 2007; Wicks et al., 2006) as well as in animal models of asthma (Sugiura et al., 2007). An increase in the number of these cells could have affected the overall phenotypic behavior of the smooth muscle cell culture. Insofar as it is not possible to effectively separate out myofibroblasts from smooth muscle cells during cell isolation and culture, and myofibroblasts stain equivalent cell markers as ASM cells, distinguishing the relative behaviors of these two cell populations was not feasible. Nonetheless, we cannot rule out that some of the differences between OVA and SAL ASM cells in response

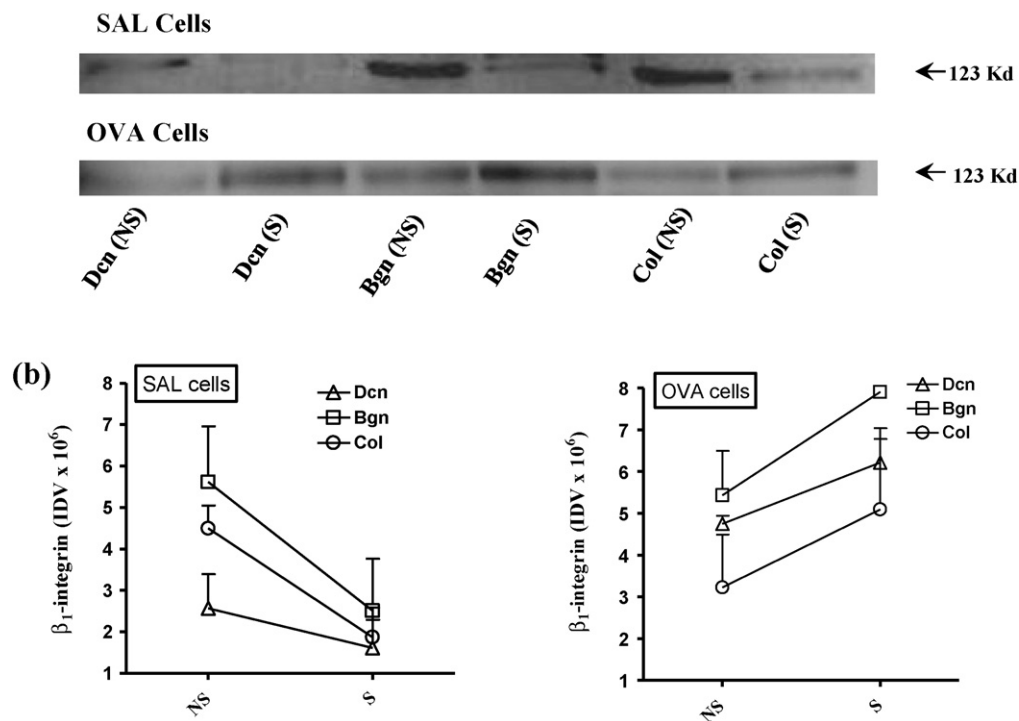
(a) β_1 -integrin

Fig. 5. Western blot analysis for β_1 -integrin expression in ASM cells subjected to mechanical strain. In SAL cells, β_1 -integrin expression after 48 h of mechanical strain (S) showed a down-regulation of β_1 -integrin relative to no strain (NS) for cells cultured on all matrices (decorin, biglycan and collagen). Conversely, strain resulted in an up-regulation of β_1 -integrin in OVA cells cultured on all matrices relative to NS (a). Densitometric analysis (b).

to matrix were related to differences in the relative proportions of myofibroblasts in the two populations of cell cultures.

We also investigated the impact of mechanical strain and ECM on growth of ASM cells. Mechanical forces are important in normal lung physiology. However, altered deposition of ECM and enhanced bronchomotor tone may lead to excessive mechanical stimulation of airways and airway resident cells. We performed *in vitro* studies in an attempt to simulate these aspects of the asthmatic airway. Rat ASM cells were cultured on matrix-coated flexible membranes and subjected to a mechanical strain regimen. Specifically, cells were exposed to an equibiaxial strain of 17% amplitude applied for 48 h at a frequency of 0.2 Hz. In order to mimic the pulsatile air-flow experienced while breathing, various studies have employed cyclical mechanical stimulation and have shown differential affects of strain in regards to ASM cell growth, function, phenotype, contractile and cytoskeletal proteins and microfilament formation and stability (Hasaneen et al., 2005; Chaudhuri and Smith, 2008; Smith et al., 1994, 1997). Our studies revealed that, relative to no strain conditions, both OVA and SAL cells responded to cyclical mechanical strain with a reduction in cell number; this was regardless of culture on decorin, biglycan or the positive control matrix, collagen. These data suggest an important role for strain in the abrogation of cell growth and survival signals. In addition, it is interesting to note that the mechanical stimulus was able to override the pro-proliferative signals normally provided by collagen (Bonacci et al., 2003; Hirst et al., 2000).

Analysis of the decrement in cell number in response to strain (relative to no strain controls), showed that, for OVA cells only, mechanical strain reduced cell number to a greater extent for cells seeded on decorin as compared to those seeded on collagen. This is noteworthy for the reason that, when seeded on plastic, OVA cells

were less affected by decorin than SAL cells. With the added stimulation of mechanical strain, OVA cells became more sensitive to the effect of decorin. While a clear explanation for this discrepancy is not obvious, some hypotheses may be offered. Differences may have resulted simply for the reason that in one set of experiments, cells were cultured on plastic, whereas for the other set of experiments, cells were cultured on silastic membranes. In addition, in the two sets of experiments, slightly different culturing protocols were employed, as initial culture proved difficult on the silastic membrane. Another plausible explanation may be that mechanical strain alters the way OVA cells would normally respond to decorin. In *in vitro* culture systems, transmission of forces occurs through the substratum, and integrins are implicated as mechanotransducers (Katsumi et al., 2005). In the current experiment, OVA cells responded to strain with an up-regulation of β_1 -integrin, regardless of the matrix examined. Integrins are transmembrane receptors consisting of non-covalently linked $\alpha\beta$ -subunits which act as the site for ECM attachments (Wang et al., 1993); the β -subunit functions as a signal transducer (Lee and Juliano, 2004). Integrins are normally associated with cell survival (Freyer et al., 2001); however, it is possible that overstretching induces signaling that results in growth suppression and apoptosis. A study by Wernig et al. (2003) showed that strain-induced β_1 -integrin activation and subsequent p38 MAPK signaling resulted in apoptosis in vascular smooth muscle cells. Other studies have shown that, depending on the cell type and stimulus, this pathway may also be capable of inducing cell survival (for review see Hsieh and Nguyen, 2005). Depending on the type of signal present, activation of various integrins and signaling pathways may occur that either promote or abrogate cell growth and survival. Interestingly, SAL cells responded to strain by down-regulating expression for β_1 -integrin.

Similarly, [Zhu et al. \(2008\)](#) have shown reduced mRNA expression of this integrin subunit following tensile mechanical forces in ligament fibroblasts. Taken together, this evidence suggests that the interplay among cell phenotype, mechanical strain, extracellular matrix, integrins and their effect on cell survival is indeed complex.

One factor that we did not investigate in the current experiment, as it was beyond the scope of this study, was the impact of the mechanical characteristics of the extracellular microenvironment, in particular the mechanical compliance of the matrix. Studies have demonstrated that matrix elasticity provides biochemical and physical cues to cells. For example, [Engler et al. \(2006, 2007\)](#) have demonstrated matrix-elasticity-dependent effects on mesenchymal stem cell shape, cytoskeletal organization, changes in gene expression and stem cell differentiation. Studies by [Mih et al. \(2011\)](#) and [Tilghman et al. \(2010\)](#) using a multiwell platform with gels of varying stiffness showed that changes in the mechanical properties of the microenvironment regulated proliferation and the morphological properties of both normal lung fibroblasts and tumor cells. It is possible that differences in the compliance of the culture system itself (plastic vs silastic) contributed to the observed differences in decorin induced OVA cell proliferation under the two experimental conditions. Future work will explore the effects of matrix compliance on growth, morphology, gene expression and protein organization of SAL vs OVA ASM cells.

A number of studies have reported the regulation of protein synthesis and release by cells in response to mechanical forces. Asthmatic fibroblasts respond to mechanical strain with an up-regulation of message for decorin and versican ([Ludwig et al., 2004](#)). In addition, ASM cells are capable of synthesizing and secreting various growth factors and pro-proliferative matrix components such as collagen (for review see [Lazaar and Panettieri, 2005](#)). In the present experiments, excessive mechanical stretch may have led to the production of excess decorin. Deposition of decorin may, in turn, be a causative factor in the reduction of cell number. Conversely, this PG may interact with strain in a synergistic manner to further inhibit cell growth. Moreover, the notion that OVA cells may be more synthetic supports our data showing that strain-induced decrements in cell number did not differ for SAL cells regardless of matrix. SAL cells may not respond to mechanical strain with enhanced decorin production, and therefore, this mechanism would not contribute to the strain-induced reduction in cell number. Finally, previous research suggesting a distinct integrin profile in ASM cells from asthmatic subjects ([Chan et al., 2006](#)) supports our data. These cells may be responding to strain and altered matrix deposition with a differential regulation or recruitment of integrins.

In conclusion, the etiology of airway remodeling is complex, involving a multitude of potential factors including alterations in matrix and the degree of mechanical strain. Changes in ASM cell number in response to altered matrix and abnormal mechanical strain, two conditions characteristic of the asthmatic airway, may have important functional implications for the pathophysiology of this disease.

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