Mechanical strain increases type I collagen expression in pulmonary fibroblasts in vitro

ELLEN C. BREEN
Division of Physiology, Department of Medicine, University of California, San Diego, La Jolla, California 92039–0623

Breen, Ellen C. Mechanical strain increases type I collagen expression in pulmonary fibroblasts in vitro. J. Appl. Physiol. 88: 203–209, 2000.—Tissue remodeling is an adaptive response to mechanical tension in the lung. However, the role of pulmonary fibroblasts in this response has not been well characterized. This study investigates the influence of extracellular matrix on the response of fibroblasts to mechanical strain. Cells were cultured on flexible-bottom surfaces coated with fibronectin, laminin, or elastin and exposed to strain. Under these conditions, fibroblasts align perpendicular to the force vector. This stimulus results in an increase in \( \alpha_1(1) \) procollagen mRNA in cells cultured on laminin or elastin but not fibronectin. Increased \( \alpha_1(1) \) procollagen mRNA was detected 6 h after exposure to strain and reached control levels by 72 h. \(^3\)H]proline incorporation into newly synthesized procollagen reflects changes in mRNA levels. Strained fibroblasts cultured on laminin or elastin incorporated 190 and 114%, respectively, more \(^3\)H]proline into procollagen than did unstrained cells. No difference was detected in strained fibroblasts cultured on fibronectin. These results suggest that fibroblasts respond to mechanical strain in vitro, and this response is signaled by cell-extracellular matrix interactions.

extracellular matrix; procollagen; remodeling

THE LUNG IS AN ORGAN in which there are large, ever changing states of mechanical tension (30). With each inspiration, the lung expands and increases tension on the load-bearing components of the lung. The large vessels and airways remain open because of radial traction, longitudinal strain is exerted on the scaffold- ing of the alveolar walls, and surface tension is generated as the lung maximally inflates. Besides controlling the physical act of breathing, mechanical tension is thought to provide regulatory signals that govern the composition of the pulmonary vasculature and airways (5). This type of stimulus is critical in the developing lung as the fetus initiates independent breathing. The mechanical act of breathing stimulates new cell proliferation, lung growth, and surfactant production (13). Furthermore, the ability to restore new lung tissue has been reported in young adults and animals after pneumonectomy (14, 28). The driving force for this response most likely stems from the mechanical signal generated by overinflation or stretch of the remaining lung (21, 28). However, an imbalance in the tensional state of the lung is found in many pathological conditions (5, 23, 35). Furthermore, mechanical ventilation commonly used in many hospital settings can result in unnatural overinflation of regions of the lung, thereby applying tension to the network of capillaries and large vessels (34). In an extreme case, a large increase in mechanical tension may ultimately lead to stress failure of the capillary wall and result in pulmonary edema and hemorrhage (36).

Pulmonary remodeling in response to hypoxic pulmonary vasoconstriction is well known to result in a thickening of the large and medium pulmonary artery walls (23). This is a result of the proliferation of smooth muscle cells and fibroblasts accompanied by increased gene expression of extracellular matrix (ECM) proteins, \( \alpha_1(1) \) procollagen and tropoelastin (22, 27). Furthermore, the direct application of circumferential tension to pulmonary arteries in vitro has also revealed increases in type I collagen and elastin (2, 15, 33) and proliferation predominantly in the adventitial fibroblast population (15). Increased mechanical strain exerted on lung parenchymal regions is also thought to result in tissue remodeling. For example, the study by Berg et al. (3) indicates that high lung inflation in vivo increases mRNA levels for ECM components and transforming growth factor-\( \beta_1 \) in the outer parenchymal region of rabbit lungs. Furthermore, in an isolated perfused lung model, it has been demonstrated that increased airway pressures or lung overinflation results in increased mRNA levels for the ECM components \( \alpha_1(1) \) and \( \alpha_2(IV) \) procollagen and laminin B chain (25). More recently, Zhang et al. (38) increased mechanical strain in unanesthetized ferret lungs by ventilation with continuous positive airway pressure over an extended period of 2 wk. Ventilation at modestly high lung volumes resulted in a 40% increase in total lung capacity associated with increased lung weight, total protein, and total cellular DNA content. These biochemical changes further support the idea that pulmonary remodeling is an adaptive response to increased mechanical strain.

In the lung, the fibroblast represents a dynamic cell type. Fibroblasts, located in the interstitial space of the alveolar septal edges throughout the lung parenchyma and within the large vessels and airways, are a high-collagen-producing cell. It is hypothesized that the pulmonary fibroblast can sense changes in mechanical tension...
EFFECT OF MECHANICAL STRAIN ON THE PULMONARY FIBROBLAST

The present study investigated the response of pulmonary fibroblasts to cyclic mechanical strain. In vitro, fibroblast cell cultures were exposed to increased mechanical tension and it was observed that collagen type I gene expression is augmented in mechanically strained IMR-90 cells. Preliminary studies also suggest that IMR-90 cells increase their level of procollagen synthesis after 1 or 24 h of exposure to increased mechanical tension. Furthermore, collagen type I gene expression is augmented in mechanically strained cardiac fibroblasts, and the mechanism of this response requires the presence of serum growth factors.

In the present study, the response of pulmonary fibroblasts in vitro to cyclic mechanical strain was further investigated. The gene expression of \( \alpha_1(1) \) procollagen in mechanically strained IMR-90 cells was evaluated at the level of mRNA and total procollagen synthesis. In addition, the influence of the ECM environment to modulate the pulmonary fibroblast response was elucidated. These findings were correlated to the proliferative state of the cell.

METHODS

Fibroblast cell cultures. The human fetal lung cell line IMR-90 (American Type Culture Collection, Rockville, MD) was routinely cultured in MEM (GIBCO BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50 U/ml). Cells were propagated in a 5% CO\(_2\)-95% air humidified incubator at 37°C. For passage, cells were released with 0.25% trypsin solution. Experiments were performed by using cells from passages 10–20.

Application of mechanical strain. The Flexercell strain apparatus (Flexcell International, McKeesport, PA) was used to expose cell cultures to increased cyclic mechanical strain. In this system, cells are grown on a flexible-bottom elastomer membrane coated with ECM protein Flex I culture plates. These culture plates were prepared by adsorption of a 1.5 M solution of fibronectin, laminin, or elastin peptides (Flexcell International). The fibronectin and elastin plates are prepared with synthesized peptides, and the elastin protein is isolated from bovine ligament by Flexcell International. For each experiment, fibroblasts were counted with a hemocytometer and seeded at an initial density of 3 \( \times \) 10\(^4\) cells/cm\(^2\). Cells were allowed to attach for 48 h. After the first 24 h, the media were exchanged for MEM containing 1% FBS.

At the initiation of cyclic mechanical strain, cells were replenished with MEM-10% FBS and placed in the Flexcell baseplate. Strained cells were exposed to a maximum 20% elongation or \(-13\) kPa pressure at a cycle of 1 stretch/s over a period of 48 h, unless otherwise stated. The Flexwell is exposed to a gradient of force that is minimal in the center of the well and maximal at the periphery. Control cells were cultured under identical conditions but remained stationary.

Morphology. Fluorescein-labeled phalloidin (Molecular Probes, Eugene, OR) was used according to the manufacturer’s directions to reveal F-actin-containing filaments. Cells were washed with PBS, pH 7.4, and fixed in 3.7% formaldehyde solution in PBS for 10 min at room temperature. Cells were again rinsed in PBS followed by permeabilization with an acetone solution at \(-20°C\) for 5 min. After a PBS rinse, cells were stained with FITC-phalloidin for 20 min at room temperature. The cells were rinsed with PBS, and the entire Flexwell culture plate was removed from the well and mounted on a slide cell side down in a 1:1 solution of PBS and glycerol. Slides were viewed immediately by fluorescent microscopy by using \( \times 25\) objective and \( \times 10\) magnification on the camera attachment and were photographed (Kodak Extrachrome 400C film, Eastman Kodak, New Haven, CT).

Northern blot analysis. Total cellular RNA was isolated by the method of Chomczynski and Sacchi (8). Two six-well Flex I culture plates were used for RNA isolation for each sample. RNA preparations were quantitated by absorbance at 260 nm, and intactness was assessed by ethidium bromide staining following separation in a 6.6% formaldehyde-1% agarose gel. Fractionated RNA was transferred by Northern blot to Zeta probe membrane (Bio-Rad, Hercules, CA). RNA was cross-linked to the membrane by ultraviolet irradiation and stored at 4°C. The blots were then probed with oligolabeled \([\alpha-\text{32P}]\text{dCTP}\) cDNA probes, which have a specific activity of at least \(1 \times 10^9\) disintegrations·min\(^{-1}\)·μg\(^{-1}\) DNA (PrimeIt II Kit, Stratagene, La Jolla, CA). The mRNA level for \( \alpha_1(1) \) procollagen was detected by using the cDNA recombinant plasmid, pr\( \alpha_1R1\), from Dr. David Rowe (10). Prehybridization and hybridizations were performed in 50% formamide, 5× SSC (20× SSC = 0.3 M sodium chloride, 0.3 M sodium citrate), 10× Denhardt’s solution (100× Denhardt’s solution = 2% Ficoll, 2% polyvinyl pyrrolidone, 2% BSA), 50 mM sodium phosphate, pH 6.5, 1% SDS, and 200 μg/ml salmon sperm DNA at 42°C. Blots were washed under high-stringency conditions of 0.1× SSC and 0.1% SDS at 65°C. The signal was obtained by exposure to XAR-5 X-ray film (Eastman Kodak, New Haven, CT) by using a Cronex Lightning Plus screen at \(-80°C\). Autoradiographs were quantitated by densitometry within the linear range of signals and normalized to 28S and 18S ribosomal RNA levels.

Assay for cell proliferation. Three wells in each of two six-well Flexwell plates were pulsed with 5 μCi/ml of \( [\text{3H}]\text{thymidine}, \) specific activity 6.7 Ci/mmol (248 GBq/mmol) (NEN DuPont) 1 h before the end of the period of applied cyclic mechanical strain. The Flexercell apparatus was then stopped, and each well was washed three times with assay medium (140 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM NaHPO\(_4\), 25 mM glucose, 25 mM HEPES/NaOH, 0.5 mg/ml BSA). Ice-cold 15% TCA (500 μl) was added to each Flexwell, and the dishes were stored at 4°C for 30 min. Wells were rinsed with water, and individual Flexwell membranes were removed from the plates and placed in scintillation vials. Scintillation fluid (Ecoscint, National Diagnostics, Atlanta, GA) was added to each vial, shaken, left in the dark overnight, and counted the next day. In the other three wells of the same plate, the total micrograms of DNA were determined by fluorometry by using Hoechst 33258 and a calf thymus DNA standard (17).

Procollagen and noncollagen protein synthesis. Procollagen and noncollagen synthesis were estimated by using the bacterial collagenase digestion assay, as previously described (24). IMR-90 fibroblasts were supplemented with \(1 \times 10^{-5}\) M ascorbic acid at the initiation of mechanical strain. One hour before the cells were harvested, the growth medium was removed, and the cells were rinsed twice with serum-free medium at 37°C. To each 25 mM well, serum-free medium containing 0.1 mM L-\( \beta \)-aminopropionitrile (Sigma Chemical, St. Louis, MO) and \(1 \times 10^{-5}\) M ascorbic acid were added, and the cells were incubated for 15 min in a 37°C CO\(_2\) incubator. The cells were then labeled by adding 30 μCi/ml of \( [\text{3H}]\text{proline}, \) specific activity 20–50 Ci/mmol (925 GBq/mmol to 1.85 TBq/mmol) (DuPont NEN) and continued to be exposed.
to cyclic mechanical strain for 1 h. This amount of [3H]proline was within the linear range of incorporation for this assay. At the end of the labeling period, the cell layer was collected. Aliquots were digested with bacterial collagenase form III (Advanced Biofactures, Lynbrook, NY) or incubated with buffer at 37°C, as described by Newman and Cutroneo (24). Total DNA levels were determined by fluorometry by using Hoescht 33258 (17).

Statistical analysis. Statistical significance was evaluated by using the Student’s t-test. P values < 0.05 were considered significant (18).

RESULTS

Morphology. Our study confirms previous reports that pulmonary fibroblasts subjected to cyclic mechanical strain undergo a strain-dependent change in morphology (6). Strained fibroblasts have a more extended shape compared with control cells and align perpendicular to the force vector in the outer periphery of the circular plate. It is in this region of the well that the force or percent elongation of the surface is greatest. Parallel to the cells, the cytoskeletal actin filaments also align perpendicular to the force vector, and this is revealed by staining the F-actin-containing microfilaments with FITC-conjugated phalloidin (Fig. 1).

Effect of matrix environment on α1(I) procollagen mRNA levels. Cells were cultured on fibronectin, laminin, or elastin and exposed to cyclic mechanical strain. On a fibronectin substratum, there was a strain-dependent decrease in the level of α1(I) procollagen mRNA (Fig. 2). In contrast, when cells were plated on a laminin matrix, there was an increase in α1(I) procollagen mRNA level in response to mechanical strain (Fig. 2). A 2.7-fold increase in mRNA levels for α1(I) procollagen was observed in mechanically strained cultures compared with unstrained, control fibroblasts. A similar response was observed when pulmonary fibroblasts were cultured on elastin. Fibroblasts cultured on an elastin matrix revealed an increase in the α1(I) procollagen mRNA level, which was greatest at an initial seeding density of 3 × 10^4 cells/cm^2 (2.3-fold). The observed difference between mechanically strained cells and control cells decreased with increasing cell densities from 4–6 × 10^4 cell/cm^2 (data not shown). Thus the potential for the pulmonary fibroblasts to increase the level of mRNA for the ECM protein α1(I) procollagen is dependent on signals from the ECM environment.

Time course of increased α1(I) procollagen mRNAs. A further examination of the time course of this response was studied in pulmonary fibroblasts, which were cultured on an elastin matrix. Cells were seeded at an initial density of 3 × 10^4 cells/cm^2, and, at various times after the initiation of mechanical strain, the cells were harvested for RNA isolation (Fig. 3). The mRNAs for α1(I) procollagen increased in mechanically strained fibroblast compared with control cells as early as 6 h after the initiation of mechanical tension. The mRNA levels for α1(I) procollagen remained increased for 48 h and returned to control levels after 72 h of mechanical stimulation. There was only a 30% increase in α1(I) procollagen mRNA levels in mechanically strained fibroblasts compared with control cells at 72 h, whereas there was a 2.3-fold increase at 12 h.

Proliferative response. The next experiment examines the dependence of increased collagen production on ongoing cell division. Pulmonary fibroblasts were again cultured at densities of 3 × 10^4 cells/cm^2 on fibronectin-, laminin-, or elastin-coated Flexwell plates.
Fig. 3. Time course of \(\alpha_1(I)\) procollagen mRNA levels in response to increased cyclic mechanical strain. Pulmonary fibroblasts seeded on an elastin matrix were exposed to increased cyclic mechanical strain (S) for 6, 12, 24, or 72 h and compared with fibroblasts cultured for same time periods in absence of strain (C). \(\alpha_1(I)\) procollagen mRNA levels were measured by Northern analysis (A) and quantitated by densitometry and normalized to 18S rRNA levels (B).

(Fig. 4). At the end of the 48-h period of increased mechanical strain, the rate of DNA synthesis was assayed by \([^{3}H]\)thymidine incorporation. On each ECM protein, there was no significant change in proliferation (Fig. 4). At the end of the 48-h period of increased mechanical strain, the rate of DNA synthesis was assayed by \([^{3}H]\)thymidine incorporation. On each ECM protein, there was no significant change in proliferation

\[\text{\textit{\&}} 1(I)\text{ Procollagen} \]
\[\text{18S rRNA} \]

\begin{align*}
\text{Hours} & \quad \text{C} & \text{S} & \text{C} & \text{S} & \text{C} & \text{S} & \text{C} & \text{S} & \text{C} & \text{S} & \text{C} & \text{S} & \text{C} & \text{S} \\
\text{6} & \quad 0 & \quad 2 & \quad 4 & \quad 6 & \quad 8 & \quad 10 & \quad 12 & \quad 14 & \quad 16 & \quad 18 & \quad 20 & \quad 22 & \quad 24 & \quad 26 \\
\text{12} & \quad 2 & \quad 4 & \quad 6 & \quad 8 & \quad 10 & \quad 12 & \quad 14 & \quad 16 & \quad 18 & \quad 20 & \quad 22 & \quad 24 & \quad 26 \\
\text{24} & \quad 4 & \quad 6 & \quad 8 & \quad 10 & \quad 12 & \quad 14 & \quad 16 & \quad 18 & \quad 20 & \quad 22 & \quad 24 & \quad 26 & \quad 28 \\
\text{48} & \quad 6 & \quad 8 & \quad 10 & \quad 12 & \quad 14 & \quad 16 & \quad 18 & \quad 20 & \quad 22 & \quad 24 & \quad 26 & \quad 28 & \quad 30 \\
\text{72} & \quad 8 & \quad 10 & \quad 12 & \quad 14 & \quad 16 & \quad 18 & \quad 20 & \quad 22 & \quad 24 & \quad 26 & \quad 28 & \quad 30 & \quad 32 \\
\end{align*}

**Fig. 4.** Proliferative response of fibroblasts exposed to increased cyclic mechanical strain. Pulmonary fibroblasts were seeded at an initial density of \(3 \times 10^4\) cells/cm\(^2\) on FN-, LN-, or ELN-coated Flexwell culture dishes. One hour before the end of mechanical stimulation period, cells were pulsed with 5 µCi/ml of \([^{3}H]\)thymidine. Incorporation of TCA precipitable counts (dpm) per microgram of total cellular DNA was determined in 6 wells. Values are means ± SE; \(n = 6\).

\[\text{[\textit{\&}H] Thymidine (dpm/µg DNA \times 10^3)} \]

\begin{align*}
\text{[\textit{\&}H] Thymidine} & \quad \text{FN} & \quad \text{LN} & \quad \text{ELN} \\
\text{[\textit{\&}H] Thymidine} & \quad 20 & \quad 30 & \quad 40 & \quad 50 & \quad 60 & \quad 70 & \quad 80 & \quad 90 & \quad 100 & \quad 110 & \quad 120 & \quad 130 & \quad 140 \\
\end{align*}

**Fig. 5.** Procollagen synthesis in mechanically strained fibroblasts. Pulmonary fibroblasts were seeded on FN-, LN-, or ELN-coated Flexwell culture dishes. One hour before the end of mechanical stimulation period, cells were pulsed with 30 µCi/ml of \([^{3}H]\)proline in presence of ascorbic acid. Incorporation of \([^{3}H]\)proline into collagen (A) and noncollagen protein (B) was determined by collagenase digestion. Values represent average counts per minute per milligram of DNA ± SE; \(n = 5–6\). Significant differences in strained cell values (S) from control (C) were observed at \(*P < 0.05\), \(#P < 0.06\), and \(\ddagger P < 0.10\).
These studies suggest a similar pattern of procollagen gene expression at the levels of both mRNA and protein synthesis in pulmonary fibroblasts exposed to increased mechanical tension.

**DISCUSSION**

Modulation of cellular collagen production by the ECM. Cell types in other organs, such as heart, bone, and kidney, have displayed the capability of responding to increased mechanical strain by altered proliferative properties and collagen production (12, 31, 37); however, very little information is known about the cellular units of the lung. In the systemic circulation, there have been reports that both cardiac fibroblasts and aortic smooth muscle cells increase the rate of collagen synthesis in in vitro models of increased mechanical tension (7, 11, 19). Furthermore, mesangial cells have demonstrated the ability to increase the expression of several ECM proteins at the transcriptional and translational levels in response to increased cyclic mechanical strain (31, 37).

However, the response of cells reported in the literature is quite variable and may be dependent on the origin and developmental age of the cellular source, as well as the cell culture conditions. Interestingly, a review of the literature points out the potential importance of signals stemming from the ECM environment. For instance, several reports of increased mechanical strain in which cell types, including cardiac fibroblasts, aortic smooth muscle cells, and mesangial cells, were stimulated while cultured on an elastin matrix resulted in a two- to fourfold increase in collagen production (7, 11, 31).

In contrast, cells plated on a collagen type I or hydrophilic matrix demonstrated much smaller changes or even a decrease in collagen production. For example, on a collagen type I substrate, pulmonary artery smooth muscle cells have a 24% decrease in collagen synthesis, and mesangial cells increase $\alpha_1$ collagen mRNA levels by 52% in response to cyclic mechanical strain. For cells plated on a hydrophilic substrate, aortic smooth muscle cells increased their collagen synthesis rate by 42%, and endothelial cells demonstrated a 50% decrease in collagen synthesis.

Comparison of published results in this way does not take into account whether the cells were fetal or adult in origin or whether they were primary cultures or established cell lines. Furthermore, the mechanical stretch apparatus, as well as the duration and amount of strain, may have differed in each experimental design. However, these experiments do bring up the question of whether signals from the ECM are important in the transduction of mechanical signals. Therefore, our study compares the response of a single cell type, the pulmonary fibroblasts, to cyclic mechanical strain in contact with a two-dimensional surface of various ECM proteins. Our results clearly demonstrate that a signal initiated at the cell surface modulates the mechanotransduction pathway, leading to a change in the gene expression of $\alpha_1$ collagen.

A similar observation in which the mechanical signal was modulated by the ECM environment was recently described by Reusch et al. (29). Neonatal vascular smooth muscle cells increase the expression of the muscle-specific sm-1 myosin in response to cyclic mechanical strain when cultured on a laminin or collagen matrix but not a fibronectin matrix. Both studies of smooth muscle cells and pulmonary fibroblasts suggest that the mechanical signal is sensed via specific ECM-cell interaction at the plasma membrane.

Strain-induced type I procollagen expression is independent of cell proliferation. Cells in culture undergo a transition from a proliferative phase in which the subset of growth-related genes, including the immediate early genes (c-fos and c-myc) and histones, is maximally expressed to a quiescent phase in which proteins characteristic of a more differentiated cell type are produced. Collagen synthesis is highest during the transitions from a sparse, actively proliferating to a confluent, quiescent cell culture (26). It is at this cellular state or log phase of growth that a mechanical strain-induced increase in procollagen gene expression was observed. The observed increase in procollagen gene expression did not correlate with an increase in the rate of cellular proliferation on any of the ECM matrices on which the fibroblasts were cultured. Interestingly, a decrease in mechanical strain-induced collagen production with increasing cell density has been reported in mesangial cells by Riser et al. (31). The ability of metabolically active fibroblasts to further increase collagen synthesis in response to mechanical strain is also supported by the requirement of serum growth factors in the mechanical strain-induced production of $\alpha_1$ collagen observed in cardiac fibroblasts (7).

Role of the ECM in transmitting a mechanical signal. The ECM environment provides important signals for many cellular functions, including morphogenesis, differentiation, angiogenesis, and remodeling (32). The ECM modifies the ability of the cell to adhere to a surface and influences cell shape. Specific focal adhesions at the cellular surface allow mechanical tension generated in the system to be transduced to the cytoskeletal network. Thus the cell is an integrated system in terms of mechanical force transduction. A change in the cytoskeletal architecture is transmitted to the nuclear matrix, ultimately allowing the expression of a subset of gene products (20). In our study, the ability of a mechanical signal to alter $\alpha_1$ collagen gene expression was modulated by contact with various ECM proteins. Whether this results in the interaction with a specific subset of adhesion molecules or a change in the number or distribution of interactions remains to be determined. In the lung, the fibroblast may be exposed to matrix environments of various compositions. For instance, in wound healing or injury, a provisional fibronectin matrix is first laid down to allow the attachment and migration of cells (9). Laminin, a major component of the basement membrane, provides an important signal for capillary morphogenesis (16). Elastin is located in the interstitial space in the alveolar septal region and in the large vessel. These are some of the main support structures in the lung that would represent load-bearing elements resistant to changes
in transmural pressure or longitudinal tension of the lung parenchyma. Future studies to elucidate the differences in the mecanotransduction pathways initiated by ECM proteins at the cell surface may give further insight into pulmonary conditions in which the balance of mechanical forces is disturbed.

I thank Dr. John B. West for critical review of this manuscript and continued support. Melanie de Guzman and Felicia Tornabene assisted in completion of the Northern analysis and procollagen synthesis experiments. This project was funded by National Heart, Lung, and Blood Institute Grant RO1-HL-46910.

Address for reprint requests and other correspondence: Ellen C. Breen, Dept. of Medicine 0623, Univ. of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093–0623 (E-mail: ebreen@ucsd.edu).

Received 31 March 1998; accepted in final form 10 September 1999.

REFERENCES


