Mechanical stretch promotes alveolar epithelial type II cell differentiation

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Mechanical stretch promotes alveolar epithelial type II cell differentiation. J Appl Physiol 91: 589–595, 2001.—Functional maturation of pulmonary alveolar epithelial cells is crucial for extrauterine survival. Mechanical distension and mesenchymal-epithelial interactions play important roles in this process. We hypothesized that mechanical stretch simulating fetal breathing movements is an important regulator of pulmonary epithelial cell differentiation. Using a Flexercell Strain Unit, we analyzed effects of stretch on primary cultures of type II cells and cocultures of epithelial and mesenchymal cells isolated from fetal rat lungs during late development. Cyclic stretch of isolated type II cells increased surfactant protein (SP) C mRNA expression by 150 ± 30% over controls (P < 0.02) on gestational day 18 and by 130 ± 30% on day 19 (P < 0.03). Stretch of cocultures with fibroblasts increased SP-C expression on days 18 and 19 by 170 ± 40 and 270 ± 40%, respectively, compared with unstretched cocultures. On day 19, stretch of isolated type II cells increased SP-B mRNA expression by 50% (P < 0.003). Unlike SP-C, addition of fibroblasts did not produce significant additional effects on SP-B mRNA levels. Under these conditions, we observed only modest increases in cellular immunoreactive SP-B, but secreted saturated phosphatidylcholine rose by 40% (P < 0.002). These results indicate that cyclic stretch promotes developmentally timed differentiation of fetal type II cells, as a direct effect on epithelial cell function and via mesenchymal-epithelial interactions. Expression of the SP-C gene appears to be highly responsive to mechanical stimulation.

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589
ages (days 18–20, full term = 21 days). The time of breeding was designated day 0. After hysterotomy, fetal lungs were dissected under sterile conditions and cleared of major airways. The tissues were finely minced and digested with 0.5 mg/ml collagenase type I and 0.5 mg/ml collagenase type IA (Sigma Chemical, St. Louis, MO) by means of vigorous pipetting for 15 min at 37°C as previously described (2). The suspension was centrifuged at 400 g for 5 min, and the pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) fetal bovine serum and sequentially filtered through 100-, 30-, and 20-µm nylon meshes. The filtrate was plated into 75-cm² flasks and incubated for 30–60 min at 37°C in an atmosphere of 95% air-5% CO₂ to allow fibroblasts to adhere. The attached fibroblasts were maintained overnight in DMEM containing 1% ITS+ (Collaborative Biomedical, Bedford, MA). Nonadherent cells were cultured overnight in 75-cm² flasks containing DMEM with 10% fetal bovine serum for attachment of epithelial cells (4).

Highly purified epithelial cells (>90%), assessed by morphological and immunocytochemical criteria, were also isolated after digestion with collagenase IA and the supernatant was aliquoted and stored at −80°C. Western blots were analyzed by the bicinchoninic acid method. Adult rat whole lung protein lysates were used as positive controls to verify specificity and sensitivity for immunoreactive SP-B. Protein samples (20 µg/lane) were separated by one-dimen- sional sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a Tris-tricine buffer system (42) and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were incubated for 1 h at 37°C in blocking buffer (Tris-buffered saline + Tween 20 with 1% bovine serum albumin) to reduce nonspecific binding. Blots were then incubated with a goat anti-SP-B primary antibody (sc-7704, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 in blocking buffer for 1 h at room temperature. After the blot was washed, secondary antibody (donkey anti-goat horseradish peroxidase, diluted 1:5,000 in blocking buffer) was added for 1 h at room temperature. Immunoreactive SP-B was detected by enhanced chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL), and specificity was verified by preabsorption with synthetic protein.

Saturated phosphatidylcholine assay. The incorporation of [methyl-3H]choline chloride into saturated phosphatidylcholine (PC) was studied in mixed type II cells and fibroblasts obtained on day 19. Cultures were maintained in the presence or absence of cyclic mechanical strain, and for the final 4 h fresh medium containing 1 µCi/ml of [methyl-3H]choline chloride was added to each well. Medium and cells were collected separately. Cellular protein content was measured using the Bradford procedure (6). Sample lipids were extracted with chloroform and methanol, the organic phase was evaporated under nitrogen, and saturated PC was assayed as previously described (38). Brieﬂy, the dried extract was re-suspended in 0.5 ml of carbonate tetrachloride containing 3.5 mg of osmium tetroxide. After 15 min, the reaction mixture was evaporated under a stream of nitrogen, and the residue was resuspended in 70 µl of chloroform-methanol (9:1, vol/vol). One-dimensional thin-layer chromatography was used to determine the pulse-labeled saturated PC production. The lipid extracts were transferred to Silica Gel H thin-layer chromatography plates (Eastman Kodak, Rochester, NY) and developed in a solvent system of chloroform-methanol-water (65: 25:4). Purified DL-dipalmitoyl-PC was used as the standard. The developed plates were stained with bromothymol blue, blotted, and vacuum-dried for 5 min at 90°C. Chromatogram
spots corresponding to the migration of saturated PC were scraped from the plates and counted by liquid scintillation spectrometry. The amount of \([\text{methyl-}^3\text{H}]\)choline chloride incorporated into saturated PC was expressed as disintegrations per minute.

**Statistical analysis.** Results (means ± SE) are expressed as the percent change from nonstretched samples. Stretched samples were compared with controls by Student’s *t*-test. *P* < 0.05 was considered statistically significant.

**RESULTS**

**Cyclic mechanical deformation of isolated type II cells increases SP-B and SP-C mRNA expression.** The effect of cyclic deformation of isolated day 18, 19, and 20 fetal type II cells on surfactant protein mRNA expression was analyzed by Northern blot (Fig. 1). We detected no effects of mechanical deformation on expression of the SP-A gene under any of the conditions studied (data not shown). On day 19 (canalicular stage), mechanical stretch increased SP-B mRNA expression by 50 ± 4% compared with unstretched controls (*n* = 3, *P* < 0.003). On days 18 (pseudoglandular stage) and 20 (early saccular stage), this mechanical stretch protocol had no effect on SP-B steady-state mRNA levels (*n* = 5, each day) compared with unstretched control type II cell cultures.

Intermittent stretch simulating fetal breathing movements produced quantitatively more robust effects on steady-state SP-C mRNA levels (Fig. 2). On day 18, mechanical deformation increased SP-C expression in isolated type II cells 150 ± 30% over expression in unstretched type II cell cultures (*n* = 3, *P* < 0.02). On day 19, mechanical deformation increased SP-C mRNA by 130 ± 30%. On day 20, there was no significant stretch enhancement of SP-C mRNA accumulation.

**Cyclic stretch of cocultured type II cells and fibroblasts markedly increases SP-C mRNA expression.** The effect of cyclic stretch on expression of surfactant proteins in mixed type II cell-fibroblast cultures (obtained from gestational days 18–20) was analyzed by Northern blot. On days 18 (*n* = 3) and 20 (*n* = 4), maintaining mixed cell cultures (1:1 cell type ratio) in the mechanically active environment did not significantly affect SP-B mRNA accumulation (Fig. 3). However, mechanical deformation of day 19 lung mixed cell cultures increased SP-B mRNA accumulation by 40 ± 2% (*n* = 4, *P* = 0.03) compared with parallel unstretched cultures (Fig. 3). Changing the ratio of epithelial cells to fibroblasts also did not alter these findings (data not shown). These results are similar in direction and magnitude to the effect of mechanical deformation on SP-B expression detected in the isolated timed-gestation type II cells. The purified fibroblast preparations did not express detectable SP-B or SP-C mRNA or immunoreactive SP-B proteins.

**Parallel day 19 coculture wells were processed for cellular protein and immunoreactive SP-B by Western blot, as described in MATERIALS AND METHODS. Mechanical deformation was associated with an overall doubling of immunoreactive SP-B (*n* = 5), although the variance among samples was sufficiently large to preclude attaining statistical significance in this series (Fig. 4).**

Figure 5 displays the effect of cyclic stretch on SP-C mRNA accumulation. Mechanical stretch of cocultures

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**Fig. 3**. Effect of intermittent stretch of isolated type II cells on SP-C mRNA accumulation. Fetal rat type II cells were isolated on gestational days 18–20 and subjected to intermittent 5% biaxial elongation at 50 cycles/min for 24h. Unstretched cells served as controls. Total RNA (10 µg/lane) was electrophoresed, blotted, and hybridized with an SP-C antisense cRNA probe. **Top:** representative autoradiograph of a Northern analysis from a control sample (left lanes) and a matched sample subjected to cyclic mechanical stretch (right lanes). **Bottom:** corresponding densities of 18S rRNA. Solid bars, cyclically stretched type II cell SP-C mRNA accumulation as a percentage of control samples (open bars). Values are means ± SE of 3–4 different experiments. *P* = 0.02; **P** = 0.03.

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**Fig. 1**. Effect of intermittent stretch of isolated type II cells on surfactant protein (SP) B mRNA accumulation. Total RNA was obtained from highly purified fetal rat type II cells (days 18–20). Cells were subjected to intermittent 5% biaxial deformation at 50 cycles/min for 24h. Unstretched cells served as controls. Total RNA (10 µg/lane) was electrophoresed, blotted, and hybridized with an SP-B antisense cRNA probe. **Top:** representative autoradiograph of a control sample (left lanes) and a matched sample subjected to cyclic mechanical stretch (right lanes). **Bottom:** corresponding densities of 18S rRNA. Solid bars, cyclically stretched type II cell SP-B mRNA accumulation as a percentage of control samples (open bars). Values are means ± SE of 3–5 different experiments. *P* = 0.003.
increased SP-C mRNA levels by 170 ± 60% on day 18 (n = 3, P = 0.02) and by 270 ± 40% on day 19 over unstretched coculture controls (n = 4, P = 0.003; Fig. 5). On day 20, mechanical stretch of cocultures did not significantly alter SP-C mRNA levels.

Cyclic stretch increases production of 3H-saturated PC. [Methyl-3H]choline incorporation into saturated PC was used as an indicator of pulmonary surfactant phospholipid synthesis. On day 19, when stretch-induced SP-B and SP-C accumulation was maximal, cyclic deformation of fetal lung cocultures increased 3H-saturated PC released into the medium by 40% over unstretched controls (n = 4, P < 0.002; Fig. 6).

DISCUSSION

The fetal lung in utero experiences two types of mechanical forces: one is generated by an active secretion of fluid by the fetal epithelium that sustains a constant transpulmonary pressure of 2.5 mmHg (50); the other is produced by “breathing” movements that cause additional intermittent deformation of 5%, similar to postnatal breathing (14). Both forces appear to be essential for lung development, including normal alveolarization (19, 52).

Distal epithelial maturation is critical for extrauterine survival. Alveolar type II cells secrete surfactant, a complex proteolipid mixture that prevents alveolar collapse at the end of expiration by lowering surface tension at the air-liquid interface. SP-A and SP-B are present in differentiated alveolar type II cells and in some bronchiolar epithelial cells (51). SP-C expression is restricted to alveolar type II cells (54). Application of tracheal ligation or drainage in utero (1, 15, 20, 25, 29) indicates that mechanical forces can modify alveolar epithelial cell phenotype. Torday et al. (45), using similar experimental conditions, demonstrated that intermittent stretch of fetal type II cells increased steady-state parathyroid hormone-related protein (PTHrP) mRNA levels. Gutierrez et al. (12) showed that, in rat adult lung explant cultures distended with a constant pressure of 21%, mechanical stretch decreased mRNA expression of SP-B and SP-C and increased a marker for the type I cell phenotype. Our results differ from those of Gutierrez et al. in the magnitude and duration of stretch, two critical factors regulating lung development and injury (22, 24, 48). Their study suggests that distension of that magnitude may shift the population of alveolar epithelial cells from type II to type I. These observations are in agreement with experimental models of fetal tracheal ligation, where overdistension of the lung produced similar effects on surfactant expression (20). Wirtz and Dobbs (53) showed that 15% constant stretch increased surfactant phospholipid synthesis in adult rat type II cells cultured on Silastic membranes. Sanchez-Esteban et al. (40) observed that 10% stretch increased SP-B expression in Clara cell-like human H441 cells. Scott et al. (43), using in vitro

Fig. 3. Effect of mechanical stretch on SP-B mRNA accumulation in cocultured type II cells and fibroblasts. Northern blots for SP-B mRNA accumulation were performed using RNA samples obtained from cocultures of separately isolated epithelial cells and fibroblasts from gestational days 18–20. Cocultures were subjected to intermittent stretch (solid bars) or were unstretched (open bars). Top: representative autoradiograph of a Northern analysis from a control sample (left lanes) and a matched sample subjected to cyclic mechanical stretch (right lanes). Bottom: corresponding densities of 18S rRNA expressed as percent change from controls. Values are means ± SE of 3–4 different litters. *P = 0.03.

Fig. 4. Effect of mechanical stretch on SP-B protein levels in fetal rat lung cocultures. Protein lysates (5 different litters, day 19) were isolated from stretched (solid bar) and control (open bar) samples and analyzed by Western blot. A: representative autoradiograph consisting of a control (C) sample and the matched stretched (S) sample. B: results expressed as percent change from unstretched controls. Values are means ± SE of 3–4 different litters.
MECHANICAL STRETCH PROMOTES TYPE II CELL DIFFERENTIATION

conditions similar to ours, found that 10% distension of fetal rabbit type II cells increased cell proliferation and surfactant phospholipid synthesis. Taken together, these studies suggest that phenotypic expression of lung epithelial cells differently responds to the degree of deformation. However, there is little information about the developmental timing of effects of mechanical forces on fetal alveolar cytodifferentiation.

In our study, we isolated fetal rat lung cells (epithelial cells and fibroblasts) obtained on each day in late gestation and studied the effects of cyclic mechanical strain using biaxial deformation and frequencies similar to fetal breathing patterns. We demonstrated that mechanical stretch of isolated type II cells increased SP-C mRNA levels by >100% and SP-B expression to a lesser, albeit significant, degree. In addition, in agreement with recent studies (11, 30), we found that SP-A expression was not stretch responsive.

The specific culture conditions were chosen to minimize dedifferentiation and phenotypic drift of the fetal type II cells. Defined, exogenous growth factor-free medium was substituted after overnight cell adhesion to prevent serum-induced loss of differentiation (32). Phenotypic stability can be maintained in serum-free isolated cell culture (9, 32) and appropriate cell density and extracellular matrix (see MATERIALS AND METHODS). The phenomenon of preserved developmentally timed differentiation and cellular responses also can be observed in rat fetal hepatocytes grown in serumless culture (10, 34).

Complex interactive and temporal-spatial communications between epithelium and mesenchyme direct the development and maturation of the embryonic lung bud into a functional air-exchanging structure. Sixty years ago, Rudnick (39) first described the importance of epithelial-mesenchymal interactions during lung development. Since then, numerous studies (16, 26, 44) have indicated that mesenchyme regulates lung morphogenesis and epithelial cell differentiation by production of growth factors (17, 37), the assembly of a unique extracellular matrix (27, 44), and direct physical contact between the mesenchymal and epithelial cells (3, 44).

The present study supports the concept that mesenchymal-epithelial signals may transduce specific developmentally timed effects on lung maturation (45). When fibroblasts were cocultured with type II cells, the magnitude of the stretch-induced effects on SP-C, but not SP-B, mRNA accumulation was further enhanced. Cyclic stretch also increased surfactant phospholipid release by ~40% in day 19 cocultures, an effect comparable in magnitude to that of glucocorticoids (49). Recently, Nakamura et al. (30), using organotypic cultures of mixed fetal rat lung cells isolated on day 19 of gestation and 5% intermittent distension, reported that transcription of the SP-C gene was enhanced by mechanical stretch. They speculated that mesenchymal regulatory mechanisms might be involved in mechanical stretch-induced SP-C gene expression. Our results emphasize the importance of such mesenchymal-epithelial interactions during this critical period of mechanically enhanced lung development.

In these studies, we did not determine the mechanism of action of cocultured fibroblasts in the mechanical response of type II cells to cyclic strain. Mechanical stretch stimulates the expression and production of PTHrP from fetal lung epithelial cells and increases the responsiveness of fetal lung fibroblasts to PTHrP (45). These effects are, at least in part, mediated by fibroblast production of leptin (46). Mechanical stretch-induced upregulation of platelet-derived growth factor-B and its receptor is another example of cell-cell communication (21). Mesenchymal-epithelial interac-

Fig. 5. Effect of mechanical stretch on SP-C mRNA accumulation in cocultured type II cells and fibroblasts. Northern blots for SP-C mRNA accumulation were performed using RNA samples obtained from cocultures of separately isolated epithelial cells and fibroblasts from gestational days 18–20. Cocultures were subjected to intermittent stretch (solid bars) or were unstretched (open bars). Top: representative autoradiograph of a Northern analysis from a control sample (left lanes) and a matched sample subjected to cyclic mechanical stretch (right lanes). Bottom: corresponding densities of 18S rRNA expressed as percent change from controls. Values are means ± SE of 3–4 different litters. *P = 0.02; **P = 0.003.

Fig. 6. Effect of mechanical stretch on \(^{3}H\)choline incorporation into saturated phosphatidylincholine (PC). Cocultured type II cells and fibroblasts (day 19) were subjected to intermittent stretch. Unstretched cultures served as controls. During the last 4 h of each experiment, the medium was changed and 1 μCi/ml of \(^{3}H\)choline chloride was added. Released \(^{3}H\)-saturated PC was assayed by thin-layer chromatography and expressed as percent change from unstretched controls. Values are means ± SE of 4 different experiments. *P < 0.05.
tions in a mechanically active environment are also modulated by direct cell-cell and cell-extracellular matrix contacts (22).

Together with our previous observation that mechanical deformation increases SP-B expression in the pulmonary adenocarcinoma H441 cell line (40), we conclude that mechanical forces can induce epithelial cell maturation by autocrine, as well as juxtacrine or paracrine, signals. To our knowledge, this is the first demonstration that mechanical stretch has a direct influence on fetal lung epithelial cell expression of SP-C and SP-B.

SP-C appears to be a useful marker for stretch-induced alveolar differentiation. From the canalicular stage of lung development, SP-C is produced only in epithelial cells lining the terminal air sacs, while SP-B is also produced in bronchiolar Clara cells (51). It is possible that different stretch responsiveness in subpopulations of SP-B-expressing epithelial cells in the region of the alveolar duct might contribute to the apparent stretch-inducible differences between SP-B and SP-C. Because it is possible that the interval for stretch responsiveness of SP-B expression might be narrower than that for SP-C, shorter durations of mechanically active culture also might be required to demonstrate more robust effects of stretch on SP-B mRNA or protein levels.

Our findings also emphasize that the effects of mechanical forces in fetal lung are highly dependent on developmental stage. The inducibility of SP-C mRNA levels was magnified from day 18 to day 19 but diminished markedly by day 20 to full term. A similar phenomenon recently was identified in chondrocytes, where mechanical stimulation of cell proliferation was found to be developmental-stage specific (55). Xu et al. (56), using organotypic culture of mixed epithelial cells and fibroblasts and 5% elongation for 48 h after 2 days in static culture, also found that strain-induced cell proliferation in fetal rat lung peaked at 19 days of gestation. These findings support the concept that the transition from pseudoglandular to saccular stages is a critical period when the distal pulmonary tissue is responsive to mechanical forces, promoting alveolarization, cell growth, and epithelial cell maturation in preparation for air breathing.

In summary, we were able to demonstrate that mechanical stretch, simulating the fetal breathing movements that normally occur in vivo, induces maturation of fetal alveolar epithelial cells. Specific effects of mechanical forces are enhanced by the presence of mesenchymal cells and are maximal during the transition from the pseudoglandular to the saccular stage of fetal lung development. We found, among the major surfactant-associated proteins, that SP-C gene expression is highly dependent on mechanical stretch. These experimental systems offer new opportunities to define the functions and signaling pathways subserved by mechanical forces during alveolar cytodifferentiation. The mechanisms underlying the mechanical stretch regulation of the SP-C gene remain to be determined.

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MECHANICAL STRETCH PROMOTES TYPE II CELL DIFFERENTIATION


