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IL-10 inhibits inflammatory cytokines released by fetal mouse lung fibroblasts exposed to mechanical stretch

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Abstract

Background—Mechanical ventilation plays an important role in the pathogenesis of bronchopulmonary dysplasia. However, the molecular mechanisms by which excessive stretch induces lung inflammation are not well characterized.

Objectives—In this study, we investigated *in vitro* the contribution of lung mesenchymal cells to the inflammatory response mediated by mechanical stretch and the potential protective role of IL-10.

Methods—Fetal mouse lung fibroblasts isolated during the saccular stage of lung development were exposed to 20% cyclic stretch to simulate mechanical injury. The phenotype of cultured fibroblasts was investigated by red oil O and alpha-smooth muscle actin staining. Cell necrosis, apoptosis and inflammation were analyzed by LDH release, cleaved caspase-3 activation and release of cytokines and chemokines into the supernatant, respectively.

Results—First, we characterized the phenotype of the cultured fibroblasts and found an absence of red oil O staining and 100% positive staining for alpha-smooth muscle actin, indicating that cultured fibroblasts were myofibroblasts. Mechanical stretch increased necrosis and apoptosis by 2- and 3-fold, compared to unstretched samples. Incubation of monolayers with IL-10 prior to stretch did not affect necrosis but significantly decreased apoptosis. Mechanical stretch increased release of pro-inflammatory cytokines and chemokines IL-1 β , MCP-1, RANTES, IL-6, KC and TNF- α into the supernatant by 1.5- to 2.5-fold, and administration of IL-10 before stretch blocked that release.

Conclusions—Our data demonstrate that lung interstitial cells may play a significant role in the inflammatory cascade triggered by mechanical stretch. IL-10 protects fetal fibroblasts from injury secondary to stretch.

Keywords

Chronic lung disease; Cytokines; Interleukin-10; Lung fibroblasts; Lung injury; Mechanical stress

Introduction

Many premature infants born with underdeveloped lungs develop bronchopulmonary dysplasia (BPD), a chronic inflammatory lung disease with serious short- and long-term complications. Although the etiology of BPD is multifactorial, mechanical ventilation-induced lung injury plays a central role [1]. Excessive stretch of the lung by mechanical

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ventilation can disrupt the integrity of the alveolar-capillary barrier, resulting in interstitial and alveolar edema. Neutrophils and macrophages recruited to the lung can then trigger and amplify an injury response by releasing cytokines and other inflammatory mediators, such as TNF- α , IFN- γ , IL-1 β , IL-6, MIP-2, MCP-1, etc [2,3]. Many of these pro-inflammatory cytokines are secreted by alveolar macrophages, fibroblasts, type II pneumocytes, and endothelial cells [4].

Distal lung parenchyma cells can be directly exposed to overstretch, and therefore to injury secondary to mechanical ventilation. It has been previously shown that mechanical stretch of type II cells releases proinflammatory cytokines [5-7]. Previous studies have also shown that type II epithelial cells are an important source of chemokines that orchestrate leukocyte migration to the peripheral lung during stimulation with LPS [8], supporting a potential important function for resident lung cells in the pathogenesis of BPD. These data support a role for parenchymal lung cells in the release of proinflammatory cytokines induced by mechanical stretch. In addition to type II cells, fibroblasts are critical for lung development and repair [9]. Fibroblasts can also secrete cytokines and growth factors and trigger an inflammatory response [10]. However, the response of fibroblasts to mechanical injury is not well defined.

IL-10 is a cytokine with established anti-inflammatory properties due to its ability to decrease the synthesis and secretion of inflammatory cytokines [11,12]. Low IL-10 concentrations have been found in the bronchoalveolar lavage of patients with BPD [13-15] and recent studies found that IL-10 expression was less prominent in the placenta of patients who developed BPD [16]. Although the ability to induce pro-inflammatory cytokines is nearly identical between preterm and term neonatal lung inflammatory cells, the production of IL-10 by lung inflammatory cells from preterm newborns is significantly reduced or absent when compared with term neonates [17]. Furthermore, a significant inverse relationship between the risk of BPD and constitutive production of IL-10 was found in neonates born between 23 and 27 wk of gestation [18]. It has been postulated that the increased levels of pro-inflammatory cytokines present in the lung of infants with BPD may reflect the inability to regulate inflammation through low expression of IL-10 [19]. *In vitro* exposure to IL-10 has been shown to have many protective effects due to reduction of the expression of pro-inflammatory cytokines in lung inflammatory cells [11,13,20]. Our group has previously shown that administration of recombinant IL-10 decreases apoptosis and release of inflammatory cytokines in fetal type II cells exposed to high magnitude of stretch [6].

Although it is widely accepted that release of proinflammatory cytokines secondary to hyperoxia and mechanical ventilation play a central role in the pathogenesis of BPD, the contribution of distal lung structural cells to the inflammatory response secondary to mechanical ventilation is not fully understood. Given that interstitial cells are directly exposed to mechanical injury, the objectives of this study were to investigate whether lung fibroblasts participate in lung injury secondary to mechanical stretch and whether IL-10 has a protective role. Our data indicate that cultured fibroblasts isolated during the saccular stage of lung development are an important source of proinflammatory cytokines and chemokines after exposure to mechanical stretch. Administration of IL-10 prior to stretch decreases apoptosis and release of inflammatory mediators.

Methods

Cell isolation and stretch protocol

Animal experiments were performed in compliance with the Lifespan Institutional Animal Care and Use Committee, Providence, RI. Fetal mouse lungs were obtained from timed-

pregnant C57BL6 mice at embryonic days 18-19 (saccular stage of lung development) and fibroblasts and type II cells were isolated as previously described [21]. Briefly, after collagenase or dispase digestion, cell suspensions were sequentially filtered through 100-, 30-, and 20- μ m nylon meshes using screen cups (Sigma). Clumped nonfiltered cells from the 30- and 20- μ m nylon meshes were collected after several washes with DMEM to facilitate the filtration of nonepithelial cells. Further type II cell purification was achieved by incubating the cells in 75-cm² flasks for 30 min. Non-adherent cells were collected and cultured overnight in 75-cm² flasks containing serum-free DMEM. For fibroblast isolation, the filtrate from 20 μ m nylon meshes was plated onto 75-cm² flasks and incubated at 37°C for 30-60 min to allow fibroblasts to adhere and maintained overnight in serum-free DMEM. After overnight culture, cells were harvested with 0.25% (wt/vol) trypsin in 0.4 mM EDTA, and plated (around 50% confluency) on Bioflex multiwell plates (Flexcell International, Hillsborough, NC) precoated with fibronectin [1.5 μ g/cm²]. Monolayers were maintained in culture for 1-2 days until they were approximately 80% confluent and then were mounted in a Flexcell FX-4000 Strain Unit (Flexcell International). Equibiaxial cyclical strain regimen of 20% was applied at intervals of 40 cycles/min for 48 hours. This regimen, which roughly corresponds to a lung inflation of 80% of total lung capacity in adult rats [22], was chosen to mimic lung cells injury. Cells were grown on nonstretched membranes in parallel and were treated in an identical manner to serve as controls.

Oil red O staining

After completion of the experiments, media were aspirated from BioFlex wells containing fibroblasts and cells were washed 3 times with 1X PBS. Cells were then covered in fixative solution containing 10% formaldehyde diluted in PBS for 30 minutes at room temperature. The fixed cells were then washed twice with 1X PBS and mounted onto slides. Fresh working solution of Oil Red O was added and the slides were incubated for 60 minutes at room temperature on a rocker. After staining, the solution was removed and the cells were washed 3 times with 1X PBS. A positive control was performed using differentiated 3T3-L1 cells.

Alpha-smooth muscle actin (α -SMA) staining

After experiments, media were aspirated and fibroblasts were washed 3 times with 1X PBS. Cells were then covered in a fixative solution containing 4% paraformaldehyde in PBS, for 20 minutes on ice. Fixed cells were then washed 3 times with 1X PBS and mounted onto slides. Cells were blocked with 3% normal goat serum (Vector Laboratories) in PBS for 60 minutes at room temperature with rocking. After blocking, slides were incubated overnight at 4°C with anti-smooth muscle actin antibody prepared in a 1:400 dilution with PBS. After primary antibody incubation, slides were washed 5 times, for 5 minutes each, with 1X TBST on a rocker. The secondary antibody (Alexa Fluor® 488 goat anti-mouse IgG) was then added in a concentration of 1:200, diluted in PBS and the slides were incubated, protected from light, for 1 hour at room temperature. The slides were then washed 3 times with 1X PBS, mounted with medium containing DAPI, and analyzed by fluorescence microscopy.

Lactate dehydrogenase assay

Quantification of cell necrosis was performed by measuring lactate dehydrogenase (LDH) release into the supernatant due to cell lysis. LDH activity was measured using a CytoTox 96 non-radioactive cytotoxicity assay (Promega) according to the manufacturer's protocol. A coupled enzymatic assay that results in the conversion of a tetrazolium salt into a red foamzan product is used to analyze LDH levels. The amount of color formed is proportional to the number of lysed cells. Absorbance at wavelength 490 nm was collected using a standard 96-well plate reader (ELx800, Bio-Tek Instruments). Fibroblasts were isolated, cultured, and stretched as described above. Before beginning the assay, a standard curve of

maximum LDH release was prepared by lysing monolayers with different numbers of cells. Once the optimal number of cells was established, a control well in which all cells were lysed by the freeze-thaw method was prepared to calculate maximum LDH release. LDH was quantified by dividing experimental LDH release by maximum LDH release (calculated after complete lysis of monolayers containing similar number of cells to sample wells).

Western blot analysis of caspase-3

Monolayers were lysed with RIPA buffer containing protease inhibitors. Lysates were centrifuged and total protein contents were determined by the bicinchoninic acid method. Equal amount of protein lysate samples (20 µg) were fractionated by NU-PAGE Bis-Tris (4–12%) gel electrophoresis (Novex, San Diego, CA) and transferred to polyvinylidene difluoride membranes. Blots were hybridized with polyclonal antibody against the 17/19-kDa cleaved caspase-3 (Cell Signaling Technology, Beverly, MA) to detect activated caspase-3. Secondary antibody was conjugated with horseradish peroxidase; blot was developed with an enhanced chemiluminescence (ECL) detection assay (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were then stripped and reprobed with antibodies to GAPDH (to control for protein loading) and processed as described before. The intensity of the bands was analyzed by densitometry.

Concentration of cytokines in the supernatant

After experiments, the cell culture medium was collected and stored at -80°C before analysis. Cell monolayers from the BioFlex plates were lysed with ice-cold RIPA buffer (150 mM NaCl, 100 mM Tris base, pH 7.5, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 3.5 mM Na₃VO₄, 2 mM PMSF, 50 mM NaF, 100 mM sodium pyrophosphate) with protease inhibitors (10 µg/ml leupeptin, 10 µg/ml aprotinin, 143.5 µM aminoethyl benzenesulfonyl fluoride). Lysates were centrifuged and total protein content was determined by the bicinchoninic acid method. Concentration of cytokines in the supernatant was measured by ELISA using a BioPlex custom cytokine detection kit (Bio-Rad) and included the following cytokines: IL-1β, IL-6, IFN-γ, KC, MCP-1, RANTES, and TNF-α. The kit was used according to the manufacturer's protocol and results were normalized to the cell lysate concentration in each sample as a representation of the number of cells added to the wells. The BioPlex kit has the following detection ranges for each cytokine: IL-1β (2.9 – 47501 pg/mL), IL-6 (1.04 – 17037 pg/mL), IFN-γ (1.7 – 27833 pg/mL), KC (3.34 – 54780 pg/mL), MCP-1 (2.94 – 48206 pg/mL), RANTES (0.91 – 14966 pg/mL), and TNF-α (3.01 – 49254 pg/mL). All reported data fell within the detection limit of the assay. The assay was conducted in an identical manner for supernatants from type II cells.

Gene Expression of IL-10 Receptors

Total RNA was isolated as previously described [23] and purified further using the Turbo DNA-free kit (Ambion). One microgram of total RNA was reverse-transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Pre-designed TaqMan® IL10Ra (Mm00434151_m1) and IL10Rb (Mm00434157_m1) primers were purchased from Assays-on-Demand™ Gene Expression Products (Applied Biosystems). To amplify the cDNA by qRT-PCR, 2 µl of the resulting cDNA were added to a mixture of 10 µl of TaqMan Gene Expression Master Mix (Applied Biosystems) and Assays-on-Demand™ Gene Expression Assay Mix containing forward and reverse primers and TaqMan labeled probe (Applied Biosystems). Standard curves were generated for each primer set and housekeeping gene GAPDH. Linear regression revealed efficiencies between 96 and 99%. Therefore, fold expressions of stretched samples relative to controls were calculated using the $\Delta\Delta C_T$ method for relative quantification (RQ) as previously described [24]. Samples were normalized to GAPDH. The reactions were performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) with the following

parameters: 50°C – 2 min, 95°C – 10 min, and 45 cycles of 95°C – 15 s, 60°C – 1 min. All assays were performed in triplicate.

Statistical analysis

Results are expressed as means $\pm$ SEM from at least 3 experiments, using different litters for each experiment. Data were analyzed with ANOVA followed by post hoc tests, and Instat 3.0 (GraphPad Software, San Diego, CA) was used for statistical analysis; $P < 0.05$ was considered statistically significant.

Results

Phenotype characterization of the fetal lung mesenchymal cells

Given that the phenotypic characteristics of interstitial fibroblasts are critical to determine the nature of signaling communication between the mesenchyme and the epithelium [25]; we determined first whether isolated fibroblasts express lipofibroblast or myofibroblast phenotype and whether a particular phenotype is affected by mechanical stretch. As shown in Figure 1, mouse fibroblasts isolated on E18-19 of gestation and placed on culture for 3-5 days showed no positive staining for lipid droplets (B) but exhibited 100% staining for α -SMA (C) indicating a myofibroblast phenotype. Mechanical stretch for 48h did not change fibroblast's phenotype compared to unstretched samples (D). Figure 1A is a positive control for lipid droplets staining. These data clearly indicate that the phenotype of the fibroblasts in our experimental system is myofibroblast.

Fetal fibroblasts express IL-10 receptors and are downregulated by mechanical stretch

Next, we investigated whether fetal fibroblasts express IL-10 receptors and the effect of mechanical stretch on gene expression of both receptors. As shown in Figure 2, fibroblasts isolated from the saccular stage of lung development express both IL-10 receptors. Mechanical stretch for 48h downregulated IL-10 receptor A by 80% compared to controls (1 ± 0.10 versus 0.21 ± 0.1). IL-10 receptor 2 decreased by 50% after stretch (1 ± 0.04 versus 0.53 ± 0.19). These data show the presence of both receptors in fetal fibroblasts and their downregulation by mechanical stretch.

Stretch-induced increase of fibroblast cytotoxicity is not affected by IL-10 administration

Previous studies from our laboratory showed that 20% cyclic stretch of fetal type II cells can damage the cell membrane and lead to cell lysis [6]. Therefore, we investigated whether fetal fibroblasts, under similar experimental conditions, responded differently. Isolated E18-19 fibroblasts were exposed to 20% cyclic stretch for 48h; cell death was analyzed by LDH release into the supernatant. These studies revealed that 20% stretch for 48h increased LDH release into the supernatant by 2-fold, when compared to unstretched samples (0.31 ± 0.05 versus 0.64 ± 0.08). Preincubation of cells with recombinant IL-10 [300 ng/ml] did not affect LDH release under control or stretch conditions, compared to samples without IL-10 (0.30 ± 0.03 versus 0.62 ± 0.07) (Figure 3). These data demonstrate that IL-10 does not protect against cell lysis induced by excessive stretch.

IL-10 decreases apoptosis in fetal fibroblasts exposed to mechanical stretch

Apoptosis plays a key role in remodeling of the lungs secondary to mechanical ventilation [26-28]. Therefore, we investigated whether mechanical stretch induces apoptosis in fibroblasts isolated from the saccular stage of lung development. Our data show that 20% cyclic stretch for 48h increases cleaved caspase-3, a key executioner protein in apoptosis, by 3-fold (0.20 ± 0.02 versus 0.59 ± 0.12). In contrast in cell monolayers incubated with IL-10, no differences were observed between control and stretch samples (0.36 ± 0.09 versus 0.41

± 0.15) (Figure 4). These data show that IL-10 protects against stretch-induced apoptosis of lung fibroblasts.

IL-10 inhibits release of pro-inflammatory cytokines and chemokines in fibroblasts exposed to mechanical stretch

In order to clarify the role of fibroblasts in the inflammatory response secondary to lung injury, we determined the level of cytokines released from fibroblasts during stretch. Culture supernatants were collected and analyzed by a custom cytokine ELISA kit, as described in Methods. Preliminary experiments determined that release of cytokines was maximal after 48h of mechanical stretch. Our results show that mechanical stretch increases release of IL-1 β into the supernatant by 1.6-fold when compared to control samples (90 ± 12 vs 141 ± 11). MCP-1 increased by 2.4-fold (298 ± 45 vs 704 ± 137); RANTES by 1.7-fold (323 ± 58 vs 554 ± 73); IL-6 by 2.3-fold (1344 ± 185 vs 3070 ± 817); KC by 2.4-fold (606 ± 68 vs 1479 ± 340) and TNF α by 1.5-fold (69 ± 3.6 vs 107 ± 3.5). In contrast, mechanical stretch did not increase release of IFN γ (24 ± 3.5 vs 30 ± 3.4). Interestingly, incubation of fibroblasts with recombinant IL-10 significantly decreased the release of pro-inflammatory cytokines and chemokines, when compared to stretched samples without IL-10 (Figure 5). The dose of IL-10 was chosen based on preliminary experiments and previous studies performed in fetal rat type II cells [6]. These data demonstrate that fetal fibroblasts actively participate in the inflammatory injury mediated by stretch and IL-10 can inhibit release of cytokines and chemokines by mechanical stretch.

Effect of mechanical stretch on cytokine levels in supernatant from type II cells

Previous experiments in adult and fetal rat type II epithelial cells have shown that stretch stimulates release of chemokines and cytokines [5-7]. Therefore, in order to establish whether mouse cells respond similarly, we tested the effect of 20% stretch for 48 hours on murine fetal type II epithelial cells. Culture supernatants were collected and analyzed by ELISA as described above. Our results in Figure 6 revealed no changes in the levels of the pro-inflammatory cytokines IL-1 β , TNF α , KC, MIP-2 and IFN γ , released from type II cells in response to mechanical stretch. Although RANTES and IL-6 showed modest increases, these results contrast with those obtained with cultured fibroblasts.

Discussion

The main findings of this study are that fetal lung fibroblasts may play a role in the injury secondary to mechanical stretch by increasing apoptosis and releasing pro-inflammatory cytokines and chemokines. Importantly, administration of IL-10 prior to overstretching decreases apoptosis and release of cytokines.

Communications between interstitial fibroblasts and the developing lung epithelium are essential for normal lung development and repair [9]. Disruption of these communications may affect lung development and contribute to the pathogenesis of BPD [25]. An important factor regulating the crosstalk between the epithelium and mesenchyme is the phenotype of the interstitial cells. Lipofibroblasts are located in the alveolar interstitium and are recognized by its characteristic lipid droplets. They participate not only in the synthesis of extracellular matrix proteins but also in the growth of the epithelium, differentiation of type II cells and alveolarization [9]. On the other hand, myofibroblasts play a larger role in tissue repair and regeneration and are the predominant phenotype in chronic lung disease [29]. Previous studies have suggested that during specific developmental stages and in response to injury, interstitial fibroblasts differentiate into a lipogenic or myogenic phenotype and can transdifferentiate from one phenotype to the other [30,31]. Given that the phenotype of the interstitial fibroblasts is of central importance in determining the nature of signaling between

the mesenchyme and the epithelium, we determined first the phenotype of the fibroblasts in our experimental system. Previous studies have found that hyperoxia augments pulmonary lipofibroblast-to-myofibroblast transdifferentiation [25]. We hypothesized that mechanical stretch would have similar effect. However, we were unable to identify fibroblasts containing lipid droplets in cultured cells. In fetal rat lungs, fibroblast lipid accumulation has been shown to increase 4 to 5-fold between days 18 and 22 of gestation and the presence of lipofibroblasts was observed in newborns immediately after isolation [25]. The absence of lipofibroblasts could be explained by the lack of adhesion to the plates during the isolation process. Another explanation would be transdifferentiation of lipofibroblasts into myofibroblasts phenotype after being in culture for three days, as previously reported [25]. In either case, our investigations provide novel information on the response of cultured myofibroblasts to mechanical injury. In the future, it would be interesting to determine whether lipofibroblasts would show similar response to overstretching and whether maintenance of a lipofibroblast phenotype would be important to minimize lung injury secondary to mechanical stretch.

Mechanical forces can disrupt lung cells membranes and cause cell death via necrosis [32]. Previous studies have shown that excessive stretch of adult and fetal type II cells can damage the cell membrane and lead to cell death [33,34]. Recent experiments from our laboratory conducted on fetal rat type II cells also showed that LDH levels increased by 50% after 24 hours of 20% stretch [6]. Our studies here revealed an increase in fibroblast lysis after 48 hours of mechanical stretch and this increase was not affected by pre-administration with IL-10. These results are supported by previous investigations from our laboratory showing that IL-10 does not protect against plasma membrane stress failure [6]. Because IL-10 decreased the release of cytokines after stretch and cytotoxicity was not affected in the presence of IL-10, we reason that release of cytokines by stretch is not caused by cell lysis.

Our data show that excessive stretch of fetal fibroblasts increases apoptosis. Our results are in agreement with previous studies demonstrating that a high percentage of stretch induces apoptosis in type II cells [6,33,35]. Increased apoptosis of interstitial cells was previously observed in fetal rabbits after tracheal ligation [36] and in human fetal lung explants after hyperoxic injury [37]. Apoptosis and impaired septation is also a prominent histopathologic finding in newborn mice exposed to mechanical ventilation [26-28]. All together, these studies indicate that although a low level of mechanical stretch may be important for interstitial remodeling during lung development [38], a higher magnitude of stretch may impair normal lung architecture. Another important finding from these investigations is the protective role of IL-10 in stretch-mediated apoptosis. Given the key role played by fibroblasts in lung development in general, and alveolarization in particular, we speculate that one of the potential mechanisms by which mechanical stretch impairs alveolar septation is by increasing apoptosis of fibroblasts. IL-10 could potentially have a therapeutic role.

Inflammation is central in the pathogenesis of BPD. Neutrophils and macrophages recruited to the lung release cytokines and other inflammatory mediators [2-4]. Therefore, most of the current investigations are focused on the response of the immune cells to lung injury. However, and as discussed in the introduction section, distal lung parenchyma cells can also be directly exposed to exaggerated stretch during mechanical ventilation. Our investigations clearly show an inflammatory response of fibroblasts to mechanical injury. The levels of all cytokines and chemokines tested, except for IFN- γ , were increased in the supernatant after mechanical stretch. In contrast, fetal lung mouse type II cells did not respond to excessive stretch with increased release of inflammatory cytokines, as previously shown [5-7]. This apparent discrepancy could be explained by the different conditions among experiments including different species and cell types, length and percentage of stretch, etc. Very few *in vitro* studies have addressed the participation of fibroblasts in lung injury [39,40] despite

evidence that interstitial cells are able to synthesize cytokines [10]. The mechanisms of cytokine release after mechanical injury have yet to be fully established; however, mechanical strain has been shown to release IL-8 in airway smooth muscle cells via the ERK and p38 pathways [41]. Additionally, the transcription factor NF- κ B has been shown to be activated by mechanical ventilation, and this activation was shown to trigger the release of pro-inflammatory mediators [42]. Although the results from these studies need to be validated in other models, our data suggest that fibroblasts may actively participate in the orchestration of the inflammatory response after mechanical injury.

Another important observation from our studies is that IL-10 was able to suppress release of proinflammatory cytokines after mechanical stretch. IL-10 administration was found to decrease the pro-inflammatory cytokines IL-1 β , IL-8 and MIP-1 α in cultured macrophages and neutrophils isolated from newborns [13,43,44]. Our group has also shown the protective effect of IL-10 in cultured fetal rat type II epithelial cells exposed to mechanical stretch [6]. In animal models of acute lung injury, administration of IL-10 reduced inflammation [45] and was associated with improved outcome [46]. Recent data also show that inhaled IL-10 reduced pro-inflammatory cytokines and mortality in an adult rat model of ventilator-induced lung injury [47]. All these *in vitro* and *in vivo* data indicate that IL-10 may have an important protective role in lung injury by modulating the inflammatory response.

In summary, these studies show that excessive stretch of lung fibroblasts isolated during the saccular stage of lung development increases apoptosis and release of proinflammatory cytokines, suggesting that myofibroblasts may have an important role in the inflammatory cascade after mechanical injury. Although the data presented here need to be validated with *in vivo* models, our *in vitro* studies suggest that IL-10 may be a promising therapeutic strategy to prevent and/or minimize lung injury secondary to mechanical ventilation in premature infants susceptible to BPD.

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Figure 1. Cultured fibroblasts express myofibroblasts phenotype

E18-19 fibroblasts were isolated and processed as described in methods, and then stained for lipid droplets using the oil red O technique (A and B). Arrows in A show 3T3-L1 cells positive for lipid droplets. B is a contrast phase of cultured fibroblasts overlaid with oil red O staining demonstrating the absence of lipid droplets inside the fibroblasts. C and D are representative fluorescence microscopy pictures showing that all cultured fibroblasts are positive for alpha-SMA (green); nuclei were counterstained with DAPI (blue). C is a control sample; D is a stretched sample. 20X magnification.

Figure 2. IL-10 receptors are present in fetal fibroblasts and downregulated by mechanical stretch

E18-19 fibroblasts were exposed to 20% cyclic stretch for 48h. RNA was isolated and reversed-transcribed, and the cDNA products for IL-10 receptor A and B were analyzed by quantitative RT-PCR using the $\Delta\Delta C_T$ method for relative quantification. Upper panels show representative amplification plots. Data on the lower panels are from four different experiments. * $P < 0.004$ vs control.

Figure 3. Effect of stretch on fibroblast cytotoxicity

E18-19 fibroblasts were exposed to 20% cyclic stretch for 48 h. Cell lysis was assayed by LDH released into the supernatant. Results are expressed as experimental minus background LDH release divided by maximum LDH release. Results are the mean $\pm$ SEM from three different experiments. * $P < 0.01$, relative to their respective controls.

Figure 4. Effects of stretch and IL-10 on apoptosis

Fetal fibroblasts were exposed to 20% cyclic stretch for 48h. Apoptosis was investigated by Western blot using an anti-cleaved caspase-3 antibody. In some samples, fibroblasts were incubated with 300 ng/ml of mouse recombinant IL-10. Membranes were stripped and reprobed with GAPDH to control for protein loading (*upper panels*, representative blot). Data in the *lower panel* are from five independent experiments. * $P < 0.05$, relative to control.

Figure 5. IL-10 decreases stretch-induced release of proinflammatory cytokines and chemokines Fetal fibroblasts were exposed to 20% stretch for 48 h in the presence or absence of rIL-10 [300 ng/ml]. Unstretched cells served as controls. Supernatants were collected and processed to assess IL-1 β , MCP-1, RANTES, IL-6, KC, TNF α and INF γ concentrations by ELISA, as described in Material and Methods. Values are mean $\pm$ SEM from 3-5 different experiments. * P < 0.05, relative to control. ** P < 0.05, xrelative to stretch + IL-10.

Figure 6. Effect of mechanical stretch on cytokine release by fetal type II cells exposed to stretch E18-19 type II cells were exposed to 20% cyclic stretch for 48 h. Supernatants were collected and processed to assess cytokine concentrations by ELISA. Results are expressed as the fold change over control samples, normalized to 1.