Response of Alveolar Type II Epithelial Cells to Mechanical Stretch and Lipopolysaccharide

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Introduction

Although many therapeutic strategies have been developed clinically [1–3], the mortality and morbidity associated with acute respiratory distress syndrome (ARDS) remains very high [4]. Since acute lung injury is nonhomogeneous [5], some lung areas are less affected and therefore more compliant. At the same time, ARDS patients may be in different stage (early or late ARDS). So type II pneumonocyte of ARDS patients may stay in different pathologic stages and receive different elongation, especially in mechanical ventilation.

IL-8 is a chemoattractant and activator of neutrophils and can contribute to the inflammatory responses associated with lung diseases [6]. Cyclic cell stretch upregulates the production and release of IL-8 by human alveolar epithelium in the absence of structural cell damage or paracrine stimulation [7, 8]. But the alveolar epithelium in different pathologic state response to stretch has never been studied.

Lipopolysaccharide (LPS) is a glycolipid that constitutes the major portion of the outermost membrane of Gram-negative bacteria. The interaction of the lipid A moiety with pattern recognition receptors results in cellular activation and the release of systemically and locally active proinflammatory molecules [9]. LPS is a major pathogenic factor potentially provoking local acute lung inflammation [10]. The human respiratory epithelium is an important environmental interface. LPS-induced epithelial responses have been reported [9].
We applied LPS to stimulate human alveolar epithelium A549, which simulate the pathogen-associated molecular pattern of acute lung injury [11]. It has been reported that alveolar type 2 cells were important in lung inflammation [12] and stretch-induced cytokine production [13]. Recognizing that all cell culture systems have limitations, the continuous human alveolar epithelial cell line (A549) was studied as a surrogate for human alveolar epithelium. A549 cells have many features consistent with alveolar type II epithelial cells. Morphologically, when grown at subconfluence, they retain a cuboidal shape, are able to synthesize lecithin and phosphatidylcholine [14] and overexpress IL-8 in response to inflammatory stimuli [15]. Cyclic cell stretch upregulates the production and release of IL-8 in human alveolar epithelium [7, 8, 13], but the effects of LPS and stretch on human alveolar epithelium have not yet been investigated in detail.

We hypothesized that the pathologic state of human alveolar epithelium may influence cell response to different mechanical stretch, which may lead to different clinical outcomes.

Materials and Methods

Cell Culture

Human type II-like alveolar epithelial cells (A549 cells) were purchased from American Type Culture Collection. Ham’s F-12K medium containing l-glutamine (2 mM), FCS (10%), and penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively; Sigma) was used as growth medium at 37°C in 5% CO₂ incubation. The cells were passaged and seeded at a density of 5 × 10⁵ cells/well on six-well culture plates (Flexcell International) before each experiment. The base of the plates consists of a flexible, collagen I-impregnated silicoelastic membrane with 25 mm in diameter. On the day of stretching, the complete medium was removed and replaced with fresh medium. Cells were stimulated with Escherichia coli LPS serotype O55:B5 (Sigma) and/or mechanical stretch.

Application of Cyclical Mechanical Stretch

Confluent A549 cells were stretched using the Flexercell® Tension PlusTM FX-4000T system (Flexcell International Corp., USA) (fig. 1), which is a computer-regulated bioreactor applying strain to the cultured cells. Through vacuum pressure, cell cultures are regulated and deformed on a collagen 1-coated culture wells when positioned on a vacuum base station. Moreover, we use BioFlex® Loading Stations (Flexcell International) to provide uniform radial and circumferential strain to a membrane surface in 5 and 15%, but not in 30% strain. Control plates are not positioned on the base station but instead are placed adjacent to it in the same incubator. All cells were maintained in 5% CO₂ at 37°C for the duration of the experiment. The cells were randomly subjected to different cyclic stretching patterns. A549 cells were stimulated with LPS (1 or 100 ng/ml) and/or mechanical stretch (5, 15, 30%) in varying frequency (0.2, 0.5, 1 Hz) at indicated time (1, 2, 4 h). Three separate cultures (n = 9) of confluent cells were used for mechanical stretching experiments.

RNA Isolation and Real-Time PCR

RNA was prepared using TRIzol® (Invitrogen, Carlsbad, Calif., USA). One microgram of total RNA was heated at 70°C for 5 min and placed on ice for 5 min. A mix of 5 μl M-MLV RT 5l reaction buffer, 10 mM of each of the four dNTPs, 200 U of M-MLV transcriptase enzyme (H–), 25 pg/μl of oligo (dT) primer and 20 U of RNase inhibitor (all from Promega, Madison, Wisc., USA) was added to each sample, followed by incubation at 40°C for 60 min and 70°C for 15 min. Real-time PCR was performed using double-stranded DNA dye SYBR Green PCR Master Mix (PE Biosystems, Warrington, UK) on the ABI PRISM 7700 system (Perkin-Elmer, Foster City, Calif., USA). PCRs were performed in triplicate and GAPDH was coamplified to normalize the amount of RNA added to the reaction. All data were analyzed using the ABI PRISM SDS 2.0 software. Primer sequences were as follows, IL-8: sense 5’ACTTTGCTAATGACGGCTGGG3’ and antisense 5’TCTCCTCTGGAGGTTT3’, GAPDH: sense 5’CGGATTG-GGCTGTATTGGG3’ and antisense 5’TCTCGCTCCTTGGA-
To compare the expression of mRNA levels among different samples, the relative expression of mRNA levels was calculated using the comparative delta C_T (threshold cycle number) method as described previously [16]. Briefly, the following formula was used: 2^-\Delta \Delta C_T, where \Delta C_T is the difference in C_T between the gene of interest and GAPDH, and \Delta \Delta C_T for the sample = \Delta C_T for the actual sample – \Delta C_T of the lowest expression sample.

### Enzyme Linked Immunosorbent Assay

According to the manufacturers’ instructions, IL-8 concentrations in the culture supernatants were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, Minn., USA).

### Statistical Analysis

SPPS 11.0 (SPPS Inc., Chicago, Ill., USA) software was used. For intergroup comparison, one-way analysis of variance was performed, and the difference was analyzed with the S-N-K test as a post-hoc analysis. For two-group comparison, we used the t test. Results are expressed as mean ± SEM (n = 9). Statistical significance was assumed if p < 0.05.

### Result

#### Effects of LPS on the Production of IL-8

Cells were subjected to 0, 1, 100 ng/ml LPS for 4 h at 5% stretch, 0.2 Hz. In the control group (without stretch), with the increasing concentration of LPS, IL-8 protein increased statistically, ranging from 75.1 ± 5.34 pg/ml (0 ng/ml LPS) to 414.07 ± 34.37 pg/ml (100 ng/ml LPS). In the presence of mechanical stretch, LPS increased IL-8 production in a dose-dependent manner. One hundred ng/ml of LPS significantly increased IL-8 production as compared with 1 and 0 ng/ml LPS (1,284.4 ± 177.16 vs. 327.07 ± 23.46 and 239.2 ± 21.51 pg/ml). IL-8 protein increased slightly in the 1 ng/ml LPS stretch group as compared with 0 ng/ml LPS, but there were no statistical differences. Comparing with each control group, mechanical stretch increased IL-8 production (fig. 2).

#### Effects of Mechanical Stretch on the Production of IL-8

Cells were subjected to 0, 5, 15, 30% stretch for 4 h at 0.2 Hz in the absence (control; no stretch) or presence of LPS (100 ng/ml). Mechanical stretch increased IL-8 production in a force-dependent manner. IL-8 concentrations in the culture medium were analyzed by ELISA. Data are means ± SEM. * p < 0.05 vs. each control group; ** p < 0.05 vs. control-only groups; † p < 0.05 vs. LPS-only groups.
Effects of Stretch Frequency on the Production of IL-8
Frequency-dependent effect of mechanical stretch was observed on IL-8 production. Cells were subjected to 5% stretch for 4 h at different frequencies in the presence and absence of LPS (100 ng/ml). With the stimulation of LPS, stretch at 0.5 and 1 Hz significantly increased IL-8 production (1,933.2 ± 110.5 and 2,058.7 ± 171.5 vs. 1,208.4 ± 90.1 pg/ml), but there were no differences between these two groups. In the absence of LPS, mechanical stretch at 0.2, 0.5 and 1 Hz had no effects on IL-8 production (291.3 ± 19.2 vs. 326.3 ± 30.4 vs. 361.4 ± 25.1 pg/ml; fig. 4).

Effects of Stretching Time on the Production of IL-8
We observed a time-dependent effect of mechanical stretch on IL-8 production. Cells were subjected to 5% stretch for 1, 2 and 4 h at 0.2 Hz. Stretch enhanced 100 ng/ml LPS-induced IL-8 production at the indicated time, especially at 4 h (4 h, 856.3 ± 69.32 vs. 2 h, 280.0 ± 68.02, and 1 h, 235.3 ± 21.36 pg/ml; fig. 5).

Effects of LPS and Stretch on IL-8 mRNA
Cells were subjected to 5% stretch for 1 or 4 h at 0.2 Hz in the presence or absence of LPS (100 ng/ml). Real-time PCR was performed to analyze mRNA levels of IL-8. LPS and mechanical stretch elevated IL-8 mRNA levels. Mechanical stretch enhanced LPS-induced IL-8 mRNA at 1 h (control group, 0.72 ± 0.09; stretch group, 1.47 ± 0.12; LPS group, 1.87 ± 0.14; stretch + LPS group, 2.4 ± 0.36) and 4 h (0.51 ± 0.10 vs. 2.51 ± 0.19 vs. 2.3 ± 0.18 vs. 3.2 ± 0.26; fig. 6).

Discussion
The Relationship between Strain and Total Lung Capacity
Strain is a measure of the degree of stretch and is expressed as the percentage of the change in cell length to resting cell length. Increases in epithelial basement membrane surface area have been reported to occur predominantly with lung inflations (residual together with tidal volume) exceeding 40% of total lung capacity (TLC) in the rat [17]. Free alveolar surface area as much as doubles when the lungs are inflated from 40% TLC to 100% TLC, some of the area change reflects unfolding of alveolar septal ‘pleats’ as opposed to extension of elastic cell and tissue elements [18]. By mathematical modeling, a change in lung volumes from 42% of the TLC to 64% of the TLC was associated with 32% of the alveolar surface area, which corresponded to 15% linear strain of alveolar cells. It is generally estimated that AECs are exposed to 1–5% strain during normal breathing, and levels of 30% strain might represent a very high tidal volume mechanical ventilation associated with ventilation-induced lung injury [19].
Clinical Data and Our Finding

The clinical relevance of experimental ventilator-induced lung injury [20] has recently received a resounding illustration by the Acute Respiratory Distress Syndrome Network trial that showed a 22% reduction in mortality in patients suffering from ARDS when lung mechanical stress was lessened by tidal volume reduction during mechanical ventilation [21]. This suggests that mechanical strain applied to the lung cells is very important in the clinical outcomes of ARDS patients.

This experiment was conducted using a cytomechanical approach. The level of IL-8 was affected by many factors, such as the passage, density, adherence of the cultured cells to collagen 1-impregnated silicoelastic membrane and the position of the seeded cells in the six-well culture plates and so on. Thus, the data were not as we had assumed. But the tendency is comparable. Mourgeon et al. [22] found that the basal MIP-2 levels in the control group varied among different experiments. In our study, we found that mechanical stretch increased IL-8 production in a force-dependent manner and enhanced LPS-induced IL-8 mRNA levels. Real-time PCR was performed to analyze mRNA levels of IL-8. Data are means ± SEM for 3 separate experiments. * * p < 0.05 vs. the control group; # p < 0.05 vs. rest of the groups at the same time point.

Cells Stimulated with Mechanical Force

In the ex vivo rat lung model, hyperventilation induced significant increases in cytokine production in the BAL fluid [25]. In the isolated perfused mouse lungs, hyperventilation-induced cytokines increased significantly in the perfusate by 30 min [26]. Numerous studies have demonstrated that mechanical ventilation can injure the lungs and produce edema and inflammation [27].

In our study, we found that 5% stain can induce IL-8 secretion consistent with the finding of Li et al. [28]. Others could only observe an increase in IL-8 release with...
30% stretch [7]. The difference may lie in the applied Flexercell 2000 system which produces a radially nonuniform strain field [29]. Therefore, only a fraction of cells in the well’s periphery were exposed to strains. Here, we used Flexercell 4000T loading station system.

Cells Stimulated with LPS and Mechanical Stretch

A synergistic effect between LPS and mechanical stretch on IL-8 production was observed after 4 h. A similar result was obtained by Vlahakis et al. [7], who found that stretch increased the effect of TNF-α on IL-8 production when A549 cells were stimulated with lower doses (0.01 and 0.1 ng/ml) of TNF-α.

In the presence of mechanical stretch, LPS increased IL-8 production in a dose-dependent manner. One hundred ng/ml of LPS significantly increased IL-8 production compared with 1 ng/ml LPS, which shows that increased IL-8 production in the alveolar epithelium depends on the cells’ pathologic state and the applied mechanical forces. In the presence of LPS, mechanical stretch increased IL-8 production in a force-dependent manner.

Salva and Waters [30] found that stretch frequency inhibits airway epithelial repair. In our study, we found a frequency- and time-dependent effect of mechanical stretch on IL-8 production in the presence of LPS. But interestingly, without the stimulation of LPS, stretch frequency had no effects on IL-8 levels. This merits further study.

There are some limitations to this research. Clearly, our in vitro model for examining the effect of stretch and LPS on alveolar type II cells eliminates the influence of complex cell-cell interactions that may modify cell response in the lung. Moreover, the pathways by which stretch regulates the secretion of cytokines from lung cells also merit further investigation.

Conclusion

Using cytomechanic methods, we found a synergistic effect of LPS and mechanical stretch on IL-8 production, which suggests that in clinical practice, when ventilation is applied to patients with sepsis, bacterial infection or other complications, this combined effect may enhance the production of cytokines, which may reversely influence the outcome of patients with ARDS.

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References


