Stretch induces cytokine release by alveolar epithelial cells in vitro

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Vlahakis, Nicholas E., Mark A. Schroeder, Andrew H. Limper, and Rolf D. Hubmayr. Stretch induces cytokine release by alveolar epithelial cells in vitro. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L167–L173, 1999.—Mechanical ventilation can injure the lung, causing edema and alveolar inflammation. Interleukin-8 (IL-8) plays an important role in this inflammatory response. We postulated that 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. To test this hypothesis, alveolar epithelial cells (A549 cells) were cultured on a deformable silicoelastic membrane. When stretched by 30% for up to 48 h, the cells released 49 ± 34% more IL-8 (P < 0.001) than static controls. Smaller deformations (20% stretch) produced no consistent increase in IL-8. Stretch of 4 h duration increased IL-8 gene transcription fourfold above baseline. Stretch had no effect on cell proliferation, cell viability as assessed by 31Cr release assay, or the release of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-α. We conclude that deformation per se can trigger inflammatory signaling and that alveolar epithelial cells may be active participants in the alveolitis associated with ventilator-induced lung injury.

MECHANICAL VENTILATION with large tidal volumes is a well-recognized cause of alveolar edema and inflammation (8). Microvascular injury, manifested by damage to the ultrastructure of endothelial cells and their detachment from the capillary basement membrane, has been demonstrated in electron-microscopic studies of hyperinflated and hyperperfused rat and rabbit lungs (8, 34). Ventilator-induced lung overdistension also disrupts alveolar epithelium, producing alveolar edema and an inflammatory lesion reminiscent of diffuse alveolar damage. In the rat model, injurious ventilation strategies induce early-response genes, such as c-fos, and promote inflammation through the release of cytokines, such as tumor necrosis factor (TNF)-α and macrophage inhibitory protein-2 (30). Although there is little doubt that mechanical ventilation can produce lung inflammation, the responsible mechanisms have not been clearly delineated.

Given this histologic evidence of alveolar tissue disruption, it might be concluded that alveolar inflammation resulting from lung overdistension represents nothing more than wound healing. As such, damage to lung cell and lung tissue structure is the critical event for initiating an inflammatory response. It is difficult to test this hypothesis in whole organ systems (to the exclusion of other proinflammatory mechanisms) because of the marked spatial and temporal heterogeneity in alveolar injury arising from the complex morphology and mechanical properties of the lung. The following experiments were designed to explore one such alternative proinflammatory mechanism, namely, mechanical strain. Specifically, we postulated that cyclic stretch of sufficient amplitude would upregulate production and release of inflammatory mediators by alveolar epithelium even in the absence of structural cell damage. This hypothesis was tested using a deformable cell culture system in which human alveolar epithelial cells (A549 cells) were exposed to cyclic stretch of varying amplitude, rate, and duration. We chose to characterize the release of interleukin-8 (IL-8) as a marker of deformation-induced inflammatory signaling because it is a potent neutrophil chemoattractant and plays an important role in the pathogenesis of acute lung injury (2, 8, 14). We report that stretch of alveolar epithelial cells increases both gene expression and release of IL-8 in a stretch amplitude-dependent manner.

METHODS

Culture of A549 pulmonary epithelial cells. Human A549 cells (American Type Culture Collection, Manassas, VA), passages 75–88, were grown on six-well culture plates (Flex I; Flexcell International, McKeesport, PA). The base of these culture plates consists of a flexible, collagen I-impregnated silicoelastic membrane with a surface area of 5 cm2 (Flexcell International). Ham’s F-12K medium containing L-glutamine (2 mM), FCS (10%), and penicillin-streptomycin-amphotericin B (100 U/ml, 100 µg/ml, and 25 µg/ml, respectively; Sigma, St. Louis, MO) was used as growth medium. A549 cells were passaged and seeded at a density of 20,000 cells/cm2 (100,000 cells/well) 48 h before each experiment.

Cell stretching device. The Flexercell 2000 cell stretching system (Flexcell International) was used to stretch A549 cells grown to a confluent monolayer as determined by light microscopy. Its principles of operation have been outlined previously (11, 25). Briefly, a computer-controlled pneumatic device intermittently exposes one side of the silicone membranes to subatmospheric pressure. The application of this transmembrane pressure results in deformation of the membrane and, in turn, the attached cells. Stretch amplitude refers to the percent change in the length of any line element in the well’s membrane. In these studies, amplitudes of 20 and 30% strain were used. Stretch was applied in a sinusoidal fashion at rates of 20 and 40 cycles/min. The strains represent maxima, as opposed to averages, within the radially nonuniform strain field produced by the Flexercell device (11).
Effects of stretch on cell proliferation rate. At time 0, 100,000 cells were seeded per well. Stretch was applied after 48 h of growth with an amplitude of 30% and a rate of 20 cycles/min. A 100-µm-deep hemocytometer (Hauser Scientific, Horsham, PA) was used to count the number of cells in 30 wells under static and 30 wells under stretched conditions. Cell numbers were compared between the two groups at 24, 48, 72, 96, and 120 h after seeding. In addition, the effect on cell proliferation of murine TNF-α alone and in combination with 30% stretch at 20 cycles/min was tested. When comparing static control A549 cells with cells with TNF-α alone, maximal doses of 10 ng/ml were used. When comparing the combined effect of TNF-α and 30% stretch, doses of 0.01, 0.1, and 1 ng/ml were used.

Effects of stretch on cell 51Cr release. To compare the functional integrity of stretched and unstretched cells, a 51Cr release assay was performed on a total of 24 wells. Cells were incubated overnight with 20 µl of 51Cr (1 µCi/µl) added to the growth medium. The following morning, the 51Cr-containing medium was discarded, the cells were washed, and the media were replaced with unlabeled medium. The amount of 51Cr released in the supernatant over the ensuing 2 h was measured using a gamma counter (Ludlum Measurements, Sweetwater, TX) and is expressed as a fraction of the radioactivity in the cell lysate (1% Triton X-100). Results were compared from three groups of wells: controls and wells that had been stretched with amplitudes of 20 or 30%.

Effects of stretch and stimulation with TNF-α on cell cytokine release. The supernatants of cells that had been stretched for 12, 24, and 48 h were analyzed for IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF; ELISA immunodetection kit; Quantikine; R&D Systems, Minneapolis, MN). The results were compared with the cytokine profiles of an equal number of time-matched static control wells. Specifically, four separate studies were performed. 1) Measurements were made on >500 wells (27 separate experiments) to compare the effects of four stretch paradigms on IL-8 secretion: amplitudes of 20 or 30% were applied at cycle rates of 20 or 40/min. 2) Measurements of GM-CSF release were confined to strains of 30%, applied 20 times/min, and their respective static controls. 3) In three separate experiments on >100 wells, the supernatant of stretched A549 cells was analyzed for human TNF-α (ELISA immunodetection kit; Quantikine; R&D Systems) to explore whether stretch augments IL-8 release through an autocrine TNF-α-dependent mechanism. 4) Different cell populations were incubated with murine TNF-α ranging in dose from 0.01 to 10 ng/ml, and their cytokine (IL-8 and GM-CSF) profiles were measured. Comparisons were made between static control and stretched cells (amplitude = 30%, rate = 20 or 40/min) at 12- to 48-h time points in the presence and the absence of TNF-α.

Effects of stretch on IL-8 gene expression using Northern analysis. IL-8 mRNA was measured after 4 h of stretch (30% amplitude at a rate of 20 cycles/min) and was compared with static and positive controls. Positive controls consisted of cells that had been stimulated with murine TNF-α (10 ng/ml) for the same time periods. Total cellular RNA was extracted using the phenol extraction method of Chomczynski and Sacchi (5). Specifically, high pure RNA isolation kits with DNase were used (Boehringer Mannheim, Indianapolis, IN). The samples were applied to a 1% agarose gel, electrophoresed, and blotted (Turboblotter; Schleicher & Schuell, Kenee, NH) to a nitrocellulose membrane (Nytran; Schleicher & Schuell). IL-8 cDNA (kind gift from Dr. T. Yoshimura, National Cancer Institute; see Refs. 17 and 18) was digested with Pst I and BamH I restriction enzymes, resulting in a 750-bp IL-8 fragment for probing. The membrane was hybridized (ExpressHyb; Clontech, Palo Alto, CA) with [α-32P]dATP and annealed to labeled human IL-8 cDNA probe (1.2 x 10⁶ counts·min⁻¹·ml⁻¹) generated by random priming (RadPrime; Gibco BRL, Grand Island, NY). The nitrocellulose membrane was then washed at a final stringency of 0.1 x SSC, 0.1% SDS at 56°C and was exposed to Kodak X-OMAT film at −70°C for 62 h. The autoradiograph was scanned and analyzed with a computer-based densitometric program (NIH Image 1.61). After the membrane was stripped of IL-8 probe, it was hybridized with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Clontech) for quantification comparison with IL-8 mRNA.

Statistical analysis. All measurements are presented as means ± SD. Statistical comparisons between experimental conditions were made with Student’s t-tests for paired observations. The number of observations and statistical comparisons among cytokine profiles refer not to the number of individual wells analyzed but to the number of matched experiments. In other words, a comparison between six stretched and six unstretched wells that had been seeded at the same time from the same passage of A549 cells is treated as an n of 1. Statistical significance was assumed at P < 0.05 with respect to a two-tailed probability distribution.

RESULTS

Effect of stretch on cell proliferation rate. Stretch with amplitudes up to 30% and rates up to 40 cycles/min did not affect cell proliferation. Figure 1 shows a comparison of cell growth curves between unstretched and cells that had been stretched by 30%, starting 48 h after plating. At no time did the number of cells differ (P > 0.3) between the two groups. TNF-α also had no effect on A549 cell proliferation regardless of whether cells were stretched or not. This was true even at the highest TNF-α concentrations used (10 ng/ml). There was, however, a trend toward decreased cell proliferation when samples remained exposed to TNF-α for >72 h (21).

Effect of stretch on cell 51Cr release. Stretch of 20 and 30% did not alter 51Cr release compared with static control cells, implying comparable cell viability. Unstretched cells released 4.7 ± 0.3% of stored 51Cr, whereas 20 and 30% stretched cells released 4.6 ± 0.1 and 5.2 ± 0.4%, respectively. The small increase in 51Cr release between cells from unstretched and 30% stretched wells was not significant. To further ensure...
cell viability, trypan blue exclusion was used to test for the presence of cell injury. All of the cells that had been stretched for 48 h excluded trypan blue, supporting a high degree of cell viability.

Effect of stretch on cell cytokine release. Compared with static controls, 30% stretch enhanced IL-8 release by A549 cells (P < 0.001) in all experiments (Fig. 2). This was true after 12, 24, and 48 h of applied stretch (Figs. 2 and 3A). Each data pair shown in Fig. 2 represents a comparison of the average IL-8 release between stretched and unstretched cells in six-well culture plates. ANOVA within each of the six wells demonstrated a coefficient of variance equal to 0.20. At the three time points, stretched cells released 48, 41, and 56% more IL-8 (P < 0.01) than matched static controls (Fig. 3A). The strain effect was similar at the two cycling rates used. When averaged across all cycling rates and durations of applied stress, 30% stretch increased IL-8 secretion by only 7.8 ± 44% compared with static controls.

Combined effects of TNF-α and stretch on cell cytokine release. A549 cells stimulated with TNF-α released IL-8 in a dose-dependent manner. At TNF-α doses of 0.01 and 0.1 ng/ml, 30% stretch enhanced IL-8 release by 22 and 24%, respectively (P < 0.05) when averaged across all time periods and cycling rates (Fig. 4). When time of stretch was analyzed separately, only at 48 h of TNF-α stimulation did 30% stretch consistently lead to an increase in IL-8 release (40 and 34%, respectively). At TNF-α doses >0.1 ng/ml, no additive effect was measured during any stretch paradigm (Fig. 4). Deformation-induced IL-8 release was not observed when the cells were stretched by 20%.

Stimulation of the cells with TNF-α at doses $\geq 1$ ng/ml for 12, 24, and 48 h induced the release of 40–80 pg/ml of GM-CSF. In contrast to its effect on IL-8,
stretch did not augment TNF-\(\alpha\)-induced GM-CSF release at any dose.

Effects of stretch on IL-8 gene expression using Northern analysis. In three separate experiments, stretch increased the measurable amount of IL-8 mRNA in A549 cells. After the application of 30% stretch for 4 h, A549 cells produced four times more IL-8 mRNA than unstretched cells (Fig. 5, A and B). In comparison, cells that were maximally stimulated with TNF-\(\alpha\) (10 ng/ml) produced 16 times more IL-8 mRNA than static controls.

DISCUSSION

The findings of this study indicate that cyclic stretch promotes IL-8 gene expression and protein release by A549 cells. This inflammatory signaling response occurred in the absence of cell injury or death, varied with stretch amplitude but not with cycling rate, and was preserved in the presence of TNF-\(\alpha\). In contrast, mechanical stretch had no effect on the release of either GM-CSF or TNF-\(\alpha\) by A549 cells and did not alter cell growth characteristics.

Numerous studies have demonstrated that mechanical ventilation can injure the lungs and produce edema and inflammation (reviewed in Ref. 8). The release of cytokines, including the neutrophil chemoattractant IL-8, is an important event in the evolution of this inflammatory lesion (2, 8, 28). However, the specific triggers for cytokine release and the cellular origins of the inflammatory mediators in ventilation-induced lung injury (VILI) have yet to be defined. In attempting to clarify these unknowns, this study suggests that pulmonary epithelial cell deformation per se may incite or augment inflammation in the absence of either cell damage or paracrine stimulation of the epithelium. It also supports a growing body of evidence that resident epithelial cells may be active participants rather than innocent bystanders in the alveolitis associated with VILI (26). Before the implications of these insights are discussed, the potential limitations of our experimental approach will be addressed.

Choice of cell model system. Recognizing that all cell culture systems have limitations (6, 9), 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 confluence, they retain a cuboidal shape, are able to synthesize lecithin and phosphatidylcholine (16), and have been found to release and/or express a variety of cytokines and growth factors, including monocyte chemotactic protein-1, IL-8 and intercellular adhesion molecule-1 (2, 15, 28). However, they have characteristic differences when compared with other commonly used culture systems, such as primary rat and human
pulmonary epithelial cells. In contrast to these primary culture systems, A549 cells secrete less phosphatidylglycerol (an important component of surfactant active material), are stimulated to secrete surfactant when exposed to cholinergic agents, and do not form the characteristic “domes” indicative of active transepithelial transport (22). On the other hand, primary culture systems are difficult to establish and may have low levels of viability and high levels of nonepithelial cell contamination. Findings from studies with animal culture systems must also be interpreted cautiously in the context of possible species differences.

In contrast to our results on A549 cells, Tschumperlin and Margulies (31) recently showed that cyclic strain of ≤25% resulted in death of rat type II alveolar epithelial cells in culture. Whether primary type II cells from a different species are responding more “physiologically” than the more spread human alveolar epithelial cell line (A549) is not clear. Type II alveolar epithelial cells cover only 5–10% of the alveolar surface and may be sheltered, in alveolar corners, from deformation injury. In contrast, type I epithelial cells cover 90–95% of the alveolar surface and in this respect may be a more relevant cell model to study deformation injury of the lung. However, establishment of primary type I epithelial cultures has met with limited success (7, 33). Therefore, investigators have used differentiated primary type II cells as a type I cell model (20). Differentiation from the type II to the type I phenotype occurs, among other factors, with time in culture. In this respect, it is of note that Tschumperlin and Margulies (31) found that alveolar epithelial cells were much more resistant to deformation injury after 5 days in culture than after 1 day.

Limitations of the stretch apparatus. A limitation of the Flexercell 2000 system is that the applied strain produces a radially nonuniform strain field (11, 25). Therefore, only a fraction of cells in the well’s periphery were exposed to strains of 20 and 30%, as was dictated by the experimental design. This introduces uncertainty about the observed mechanical threshold for IL-8 release. It must also be pointed out that in experiments with the Flexercell 2000 system, stress is not confined to the basolateral cell surface. The radial acceleration of cells relative to the stationary medium and “swishing” of the medium produces shear forces over the apical cell surface of a yet-to-be-defined biological significance. After these experiments were performed, cell strain devices with uniform radial stretch became commercially available (25) and offer an opportunity to control and refine deformation paradigms.

Despite the limitations of the Flexercell 2000 system, IL-8 release from alveolar epithelial cells varied with strain amplitude. Peak stretch of 20% produced no measurable increase in IL-8 secretion, whereas the effects of 30% stretch on IL-8 signaling could be invariably demonstrated. There was no radial gradient in trypan blue exclusion, arguing against heterogeneity in cell viability with applied stress. In fact, the more sensitive chromium release assay suggested no impairment in cell integrity. We have no explanation for why cycling rate had no effect on cytokine release except to speculate that the ability of cells to respond to deformation may have been limited or that the range of rates examined was too small.

Scaling of cell stretch in vitro to alveolar cell deformation in vivo. The amplitude threshold in stretch-induced inflammatory signaling raises an important question: how does the stretch we have applied to A549 cells relate to the deformation experienced by alveolar epithelium in situ? Envisioning the lung as a homogeneous, isotropic elastic solid, one predicts that the length of any element must change with volume to the one-third power. Because lung volume more than doubles during an inspiratory capacity maneuver, it is not unreasonable to think that some regions of the injured lung would be strained by 30% during mechanical ventilation at currently recommended ventilator settings (27). It is these levels of lung strain that are implicated in ventilator-induced lung injury. Actual in situ parenchymal strain measurements in recumbent normal dogs yielded values as high as 40% when the airway pressure was increased from 0 to 30 cmH2O (23). In these studies, the parenchymal marker technique (3) was used so that strains could be computed for tissue volumes as small as 1 cm³. It is uncertain, however, whether the continuum mechanics assumptions that were used in these calculations are valid on a scale of a few micrometers, i.e., the scale of a single cell.

Classic morphometric studies on excised, perfusion-fixed rabbit lungs emphasize the importance of volume history and the delicate interplay between surface tension, surface area, tissue properties, and capillary pressure on alveolar geometry (1, 18, 35). Although the free alveolar surface area as much as doubles when the lungs are inflated from 40% total lung capacity (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 (1). Consequently, the epithelial basement membrane area may increase by only 50%, corresponding to a strain of 22%. All in all, currently available data on the micromechanics of the lung, although not definitive, place the degree and distributions of strains that we have examined in vitro within the range of strains one might reasonably expect during mechanical ventilation in vivo.

Stretch-induced cytokine responses and signaling in A549 cells. Mechanical strain has been found to cause changes in morphology, alterations in cell cycle, and modification of DNA synthesis and protein production in many different cell systems (4, 13). The mechanisms and molecular pathways governing mechnochemical signaling are numerous and have been well reviewed (24, 32). The “downstream” effects of mechanical forces in the lung are becoming more apparent, as Wirtz and Dobbs (36) have shown by delivering a single stretch to type II rat epithelial cells, resulting in calcium mobilization and surfactant production. However, mechanisms of cause and effect in mechanotransduction are not clear. Cellular tensility predicts that external mechanical forces are conducted from the extracellular matrix leading, through the cytoskeleton, to physical
nuclear changes and thus protein modification (13). It also seems clear, however, that a vast array of cytosolic second messengers and protein kinases are integrally involved in transduction mechanisms. How these two pathways interact has not been clearly established and is still under active investigation. Direct measures for transduction pathways and proteins were not undertaken; therefore, we can only speculate on the mechanism of mechanotransduction in our experimental system. Regardless, it is clear from these studies that mechanical stretch does exert an effect at the level of gene transcription, although mRNA instability and posttranscriptional modifications may also have played a significant role. Application of cyclic stretch over 4 h promoted IL-8 gene transcription in A549 cells. A similar time course in gene transcription activity had been observed when TNF-α was used to stimulate A549 cells (15). Maximal doses of TNF-α exerted about a fourfold greater effect on IL-8 gene transcription than did mechanical stretch.

The effect of stretch on IL-8 protein release was preserved in the presence of TNF-α, a powerful proinflammatory signaling molecule that causes IL-8 gene expression and protein release by A549 cells (15, 28). At doses of >0.1 ng/ml, TNF-α and 30% stretch appeared to have a modest additive rather than a synergistic effect on IL-8 release. However, this additive effect was overwhelmed when the cells were maximally stimulated with TNF-α (10 ng/ml). It is unclear whether one should characterize the stretch effect as marginal or a TNF-α concentration of 10 ng/ml as unphysiological. Of note, TNF-α concentrations in bronchoalveolar lavage fluids of patients with acute respiratory distress syndrome tend to be in the picomolar as opposed to the nanomolar range (12). At these lower concentrations, the two stimuli (stretch and TNF-α) were clearly additive. Human TNF-α could not be identified in the supernatant of either stretched or control cells. We conclude that deformation-induced IL-8 signaling is not regulated by an autocrine TNF-α-dependent mechanism.

Although our studies have focused largely on IL-8 as a marker of deformation-related inflammatory signaling by alveolar epithelium, the absence of a stretch effect on GM-CSF release suggests at least some degree of specificity in mechanoresponses of A549 cells. GM-CSF is a glycoprotein cytokine that enhances neutrophil responsiveness to chemotactic factors, increases neutrophil migration to sites of inflammation, and is considered important for antigen recognition by dendritic cells (10, 29). A549 cells did release GM-CSF when stimulated with TNF-α, although much larger doses (>1 ng/ml) were required than had been necessary to induce IL-8 release. Also in contrast to this IL-8 response, deforming stresses did not enhance GM-CSF release at any TNF-α dose.

Theroy of cell stretch in alveolar epithelial inflammatory signaling in VILI. It has been recognized only recently that alveolar epithelial cells contribute to the initiation, augmentation, resolution, and repair of inflammatory processes (2, 14, 26). In accordance with this view, the alveolar epithelium of hyperventilated rat lungs has been found to contain large amounts of mRNA for various proinflammatory cytokines, including TNF-α (A. Slutsky, personal communication). It remains unclear whether this TNF-α gene expression in alveolar epithelium of the intact overdistended lung is a primary consequence of alveolar cell deformation or the result of inflammatory signaling cascades resulting from non-specific epithelial injury, cues from alveolar macrophages, or both. Under the in vitro conditions of these experiments, stretch did not result in cell death and so did not provide a nonspecific stimulus for inflammatory upregulation. Certainly, alveolar macrophages may be stimulated to clear fibrin and exuded proteins from the alveolar space and, in turn, act as proinflammatory signalers to the epithelium. The findings presented in this paper do not speak to this directly but do underscore that epithelial cells may not require paracrine stimuli to release inflammatory mediators in response to deformation.

In conclusion, cyclic deformation of sufficient amplitude can upregulate production and release of inflammatory mediators by alveolar epithelium, even in the absence of structural cell damage. The importance of this mechanism relative to more conventional wound healing paradigms awaits further study.

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