Equibiaxial deformation-induced injury of alveolar epithelial cells in vitro

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Tschumperlin, Daniel J., and Susan S. Margulies. Equibiaxial deformation-induced injury of alveolar epithelial cells in vitro. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L1173–L1183, 1998.—Deformation of the alveolar epithelial basement membrane with lung inflation has been implicated in blood-gas barrier breakdown during the development of ventilator-induced lung injury. To determine the vulnerability of alveolar epithelial cells to deformation-induced injury, we developed a cell-stretching device that subjects cells to cyclic, equibiaxial strains. Alveolar epithelial type II cells from primary culture were tested 1 and 5 days after seeding, during which time the cells underwent major morphological and phenotypic changes. Cells were subjected to changes in surface area of 12, 24, 37, and 50%, which corresponded to lung inflation of ~60, 80, 100, and >100% of total lung capacity. Deformation-induced injury of alveolar epithelial cells, assessed with a fluorescent cell viability assay, increased with deformation magnitude and decreased with time elapsed after seeding. In cells stretched after 1 day in culture, the percentage of dead cells after a single deformation ranged from 0.5 to 72% over the range of deformations used. In cells stretched at 5 days, the percentage of dead cells ranged from 0 to 9% when exposed to identical deformation protocols. These results suggest that morphological and phenotypic changes with time in culture fundamentally change the vulnerability of alveolar epithelial cells to deformation.

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...to systematically examine the effects of physiologically relevant deformations on alveolar epithelial viability in vitro, thus eliminating many of the complicating factors inherent in animal and isolated lung studies.

Previous studies of the effect of mechanical deformation on alveolar epithelial cells have used one of two experimental systems: deformation of thin, elastic cell culture substrata by positive or negative pressure (1, 53) or elongation of foam sponges seeded with cells (30). These studies have demonstrated that cell deformation stimulates surfactant secretion in adult alveolar epithelial type II (ATII) cells (53) and surfactant-related phospholipid synthesis, increased cellular proliferation, and prostacyclin production in fetal alveolar epithelial cells (30, 42, 44). Although these studies have provided valuable information about deformation-mediated behavior in lung cells, each of the devices has important limitations that prevent a detailed correlation between deformation and functional changes. Inflation or deflation of a circular cell culture substratum subjects cells to nonuniform loading conditions, causing cellular deformation to vary dramatically with radial position (See Refs. 25, 51 for detailed analyses). The deformations applied to cells on an elongating foam sponge are also nonuniform and, more importantly, difficult to quantify. The nonuniform strain fields created with these devices preclude attributing changes in cell physiology to a single strain level and make interpretation of assays from heterogeneously deformed cell populations problematic. Finally, none of these studies has specifically addressed the effect of large deformations on alveolar epithelial injury nor have they examined cellular responses at various intervals after seeding of primary cultures, during which time alveolar epithelial cells undergo major structural and functional changes.

Therefore, the goals of this study were twofold: first, to develop and characterize a device capable of subjecting alveolar epithelial cells to uniform, equibiaxial strains up to and beyond those measured in isolated lungs and second, to determine the vulnerability of alveolar epithelial cells to equibiaxial deformation-induced injury. We describe a cell-stretching device that provides repeatable membrane deformations that are identical in all directions and uniform across the cell culture surface. In addition, we demonstrate that cell deformations are indistinguishable from those of the underlying membrane, allowing changes in cell physiology to be attributed to specific and quantifiable deformations. Using this device, we show that alveolar epithelial injury increases with strain magnitude and...
duration and decreases with cellular seeding density and time elapsed after seeding.

MATERIALS AND METHODS

Device design. The custom-designed device is schematically similar to those presented previously (26, 29, 40). A 0.2-mm silicone membrane (gloss/gloss finish; Specialty Manufacturing, Saginaw, MI) serves as the deformable substratum for cell attachment. Wells are formed by securing the silicone membrane to the bottom of a custom-designed polysulfone ring with a rubber O-ring pressed into a groove in the bottom of each ring (see Table 1 for dimensions). The device is designed to deform up to nine wells simultaneously. Each well is mounted in the cell-stretching device on a stationary top plate (Fig. 1). Membrane deformation is provided by an annular indentor that contacts the bottom of the silicone membrane near the periphery of the cell culture surface. Vertical displacement of the indentor results in sliding of the membrane over the indentor surface and stretching of the membrane in the plane transverse to the direction of indentor motion. To ensure smooth sliding of the membrane over the indentor, a thin coat of lubricant is applied to the bottom surface of the membrane (Braycote 804, Castrol). Indentors are mounted under each well on a plate parallel to the well-mounting plate. Vertical motion of the indentor plate is provided by a variable speed DC motor (Bodine Electric, Chicago, IL) attached to the indentor plate by a variable length cam and is guided by brass bushings in the four corners of the plate. Frequency and strain rate are varied by adjusting the motor speed. Stretch amplitudes and minimum strains can be set independently with the cam and linkage.

Validation of membrane deformation field. Five wells were studied individually in the device to determine the relationship between indentation depth and membrane strain, to compare radial and circumferential strains, and to determine the variability of strain over each membrane surface and from well to well. Each well was studied during cyclic deformation at five indentation amplitudes (0.25, 0.38, 0.51, 0.63, and 0.76 cm). An additional three wells were used to

Table 1. Critical dimensions in cell-stretching and modified devices used to measure cellular deformation

<table>
<thead>
<tr>
<th>Dimension</th>
<th>Cell-Stretching Device</th>
<th>Modified Device</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well ID</td>
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<td>3.810</td>
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<td>O-ring nominal thickness</td>
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<tr>
<td>O-ring nominal diameter</td>
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<td>Indentor ID</td>
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<tr>
<td>Indentor thickness</td>
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</tbody>
</table>

Values are in cm. Note preserved ratio of well ID to indentor ID of 5:4.

Fig. 1. Schematic of cell-stretching (A) and modified (B) devices used to characterize cell deformations. Both devices employ a circular cell culture substratum deformed by a circular indentor, providing uniform, equibiaxial substratum deformations within indentor radius. Actual cell-stretching device has 9 parallel indentors for simultaneous deformation of 9 wells. C: comparison of substratum deformations with indentation in both devices. Solid line, multiwell device; dashed line, modified single-well device. %ΔMembrane SA, percent change in membrane surface area. Differences are due to dimensions not scaled between the 2 devices (see Table 1).
determine stability of the strain field over 1 h of cyclic deformation at an amplitude of 0.76 cm.

The membranes were marked with an approximate center dot and five dots along four perpendicular radial spokes. While the device operated at 15 cycles/min, 3 consecutive cycles of dot motion were recorded on video at 60 frames/s. Five frames per second were captured digitally (Adobe Premiere 4.0) at a resolution such that the dots occupied a 2 × 2 pixel area, and the dot locations were digitized in a Cartesian coordinate system with image-analysis software [National Institutes of Health (NIH) Image]. The true center of each well was determined from the well periphery visible in the image, and the center was used to calculate the position of each point in polar coordinates [radial coordinate (r) and circumferential coordinate (θ)]. Radial locations of each point were scaled and corrected for membrane motion relative to the camera, determined by filming undeformable grids moving on the indentor. Lagrangian radial (ε_r) and circumferential (ε_θ) membrane strains were computed from the relationships (24)

\[ \epsilon_r = \frac{\partial u}{\partial r} \]  
\[ \epsilon_\theta = \frac{u}{r} \]  

where u is the radial displacement and r is the undeformed radial coordinate of each point. Linear regression of the displacement-radius relationship along each spoke was used to determine ε_r. Average strain amplitude was computed across the three cycles. The dependence of ε_r amplitude on θ was determined at the largest indentation (0.76 cm) by comparing ε_r across the spokes. No dependence on θ would correspond to a uniform ε_r field. ε_θ values were computed along each spoke at each of the five radial positions occupied by markers. The ε_θ amplitudes were then averaged at each radial location across the four spokes to determine the average ε_θ at each radial location. The dependence of ε_θ amplitude on r at an indentation of 0.76 cm was determined. No dependence on r would correspond to a uniform ε_θ field. The ε_r for the well was defined as the average across the four spokes, and the ε_θ for the well was defined as the average across all r values. To evaluate whether the strain field is equal in the r and θ directions (equibiaxial), these average ε_θ and ε_r amplitudes for each well were compared at each strain level. To evaluate the stability of the strain field in the three wells tested for 60 min, the average ε_r and ε_θ amplitudes for each well were computed during the first minute of cyclic stretch and after 60 min of stretch and were compared.

Design and validation of a microscope-mountable device. A modified device was constructed to determine the relationship between the cellular and membrane strains (See Table 1 for dimensions). The modified device is based on the same concept as the nine-well motor-driven device but is composed of a single unit with an annular indentor affixed to the stage of an inverted microscope (Fig. 1). The cell culture surface is deformed manually by moving the modified well down onto the indentor. The membrane thus deforms without moving out of the focal plane of the microscope, allowing visualization of the cells during quasi-static deformation.

To determine the strain-indentation relationship for the modified device, three modified wells were prepared and marked with a total of five dots (center and four equidistant orthogonal radii), then photographed with a digital camera on a photographic enlarging stand, with images captured in the undeformed configuration and at 0.106-cm increments of indentation up to a depth of 0.95 cm. Digital images were analyzed with image-analysis software (NIH Image), and the stretch ratio (λ), defined as

\[ \epsilon_r = \epsilon_\theta = \sqrt{\lambda - 1} \]  

in an equibiaxial strain field, was computed between the center and peripheral dots in each of four directions with respect to the undeformed positions with the relationship

\[ \lambda = \left( \frac{1 + \epsilon_r}{1 - \epsilon_r} \right)^{2/3} \]  

The average λ was computed across the four directions for each membrane at each indentation depth. Membrane deformations were computed as changes in MSA (%ΔMSA) rather than as ε_r and ε_θ for direct comparison with changes in cell surface area (CSA). The %ΔMSA was computed from the average λ with

\[ %\Delta MSA = (\lambda^2 - 1) \times 100 \]  

Cell culture protocol. ATII cells were isolated from specific pathogen-free male Sprague-Dawley rats weighing 180–200 g. The rats were anesthetized with pentobarbital sodium (50 mg/kg body wt i.p.). The trachea was cannulated, the lungs were mechanically ventilated, an abdominal aortotomy was performed, and the lungs were perfused via the pulmonary artery to remove blood. The lungs were then excised, and ATII cells were dissociated and isolated in a technique adapted from Dobbs et al. (11). Elastase solution (7 ml, 3 U/ml; Worthington Biochemical) was instilled into the airways of the excised lungs and incubated for 32 min, with an additional 6 ml instilled at 12 and 22 min of incubation. The lungs were finely minced in the presence of deoxyribonuclease (Sigma) with a tissue chopper. The elastase reaction was stopped with fetal bovine serum (GIBCO BRL, Life Technologies). Cell separation was done by filtration and plating of the cells on bacteriological plastic precoated with rat IgG (Sigma), with 3 mg IgG·5 ml Tris-HCl·1·plate⁻¹. After 1 h of incubation, the type II cells were isolated from the macrophages and contaminating cells by panning (11).

ATII cells were resuspended in minimum essential medium with Earle’s salts and L-glutamine (Mediatech, Fisher Scientific), supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin (GIBCO BRL, Life Technologies). Cells were plated at 1.0 × 10⁶ cells/cm² (unless otherwise noted) on Silastic membranes coated with 50 µg/ml of fibronectin (Boehringer Mannheim Biochemicals). Cell attachment was limited to the central portion of each custom-made well by placing a piece of Tygon tubing in the center of each well and seeding cells only within this restricted area. Cell purity, assessed by phosphine 3R staining of adherent cells at 1 day, was >95% (32). Wells were studied at either 1 (24 h) or 5 days after isolation. Cells were incubated in 5% CO₂ at 37°C, with medium replaced every 24 h. Measurements of cellular deformation. The wells were mounted in the modified device, and the cells were photographed just before deformation and after each 0.106-cm increment of quasi-static indentation until a total indentation of 0.95 cm was reached (>20 objective, Nikon Diaphot). Three wells were studied at each time point after seeding. Photographs were digitally scanned and analyzed with image-analysis software (NIH Image). The perimeter of six cells from each of the three wells was digitized at each indentation depth, and the enclosed CSA was calculated. Pictures at each indentation depth were compared with the undeformed cells.
to determine the percent change in CSA (%ΔCSA) according to

$$%\Delta CSA = \frac{CSA_d - CSA_u}{CSA_u} \times 100$$

where CSA_u is the undeformed surface area of each cell and CSA_d is the deformed surface area of the corresponding cell at each indentation increment.

Measurement of cellular injury. The wells were washed three times with serum-free Dulbecco's minimum essential medium supplemented with 100 U/ml of penicillin and 0.1 mg/ml of streptomycin (GIBCO BRL, Life Technologies) in which sodium bicarbonate was replaced with 20 mM HEPES. The experiments were carried out at 37°C in room air. Before deformation, ethidium homodimer-1 and calcein AM were added to the wells for final concentrations of 0.23 and 0.12 μM, respectively (LIVE/DEAD, Molecular Probes). Ethidium homodimer-1 (excitation ~495 nm, emission ~635 nm), which is excluded by the intact plasma membrane of live cells, enters cells with damaged membranes and undergoes a fluorescence enhancement on binding to nucleic acids. Calcein AM (excitation ~495 nm, emission ~515 nm) is well retained within live cells. The wells were subjected to a single deformation cycle or to 60 min of cyclic deformation, with surface area changes (%ΔSA) of either 12, 25, 37, or 50%. These equibiaxial deformations are roughly equivalent to those found in isolated rat lungs at 60, 80, 100, and >100% of total lung capacity (Fig. 2). To rule out the possibility that fluid flow over the cells induced injury, the wells were also subjected to 1 h of mixing on an orbital shaker at 1 Hz for 60 min.

One hour after the onset of the deformation protocol, control and stretched wells were examined with an inverted epifluorescence microscope (×20 objective, Nikon Diaphot), with images of the cells captured and stored with a digital-imaging system (Hamamatsu charge-coupled device camera and controller, Metamorph 2.5, Universal Imaging). Images were acquired randomly from four locations on each well, with separate filter blocks for visualization of ethidium homodimer-1 and calcein AM. Two images from each well were subsequently analyzed by counting stained cells, and the percentage of dead cells was determined. Results from unstretched control wells were subtracted from stretched wells; thus the percentage of dead cells reported is solely attributable to the deformation protocol.

Statistics. Results are presented as means ± SD. Except where noted, groups were compared by analysis of variance with the statistical package JMP (SAS Institute). Differences were regarded as significant when P < 0.05. For cell viability experiments, multiple observations from each well were averaged and counted as an experimental measurement. For each equibiaxial deformation magnitude, duration, and time after seeding studied, one to two wells from three separate cell isolations were examined, for a total of four to six wells in each group.

RESULTS

Device characterization. The cell-stretching device provides an essentially uniform strain field in which ε_i and ε_l are equivalent and neither varies significantly with location (Fig. 3). Specifically, at the largest indentation depth (0.76 cm, producing a 50%ΔMSA), the dependence of ε_i on h and of ε_l on r were determined to be not significant (P > 0.05 by Tukey-Kramer honestly significant difference test), demonstrating uniformity of the strain field. Second, the average ε_i and ε_l values were not significantly different within any membrane or indentation depth (P > 0.1 by two-tailed paired Student’s t-test) or when all the data were considered together (P > 0.1; correlation coefficient = 0.98), demonstrating an equibiaxial strain field. Furthermore, membrane strain did not change significantly after 1 h of continuous operation as determined by the comparison of strains measured before and after continuous cycling for 60 min at 15 cycles/min in 3 membranes (P > 0.1 for ε_i vs. ε_l by Student’s paired t-test). Because of the uniform and equibiaxial nature of the strain field, ε_i and ε_l at each indentation level were averaged together to determine the relationship between indentation depth and membrane strain for the multiwell device (Fig. 1C, solid line).

Membrane strains could be measured only for the portion of the membrane that remained in the horizontal plane throughout the deformation cycle. In all experiments with cells, attachment and spreading were confined to this fully characterized portion of the substratum by coating the surface and maintaining the cells within a cylinder of Tygon tubing placed in the center of the well. Cell injury was unaffected by removal of the tubing before the cells were washed at the beginning of the experimental stretching.

Cell attachment and deformation. Alveolar epithelial cells grown on fibronectin-coated Silastic membranes in polysulfone wells were photographed at 1 and 5 days after cell isolation. At 1 day (Fig. 4, control), ATII cells contained prominent lamellar bodies and were found in clumps. Although most cells appeared to be well attached to the substratum and undergoing cell spreading, a small percentage of attached cells remained rounded. At 5 days (Fig. 4, control), the cells were confluent, considerably more flattened, and lacked lamellar bodies. These findings are consistent with re-
results on tissue culture-treated plastic and polycarbonate filters (2, 45). By seeding the cells at high density, the average substratum surface area covered by each cell was maintained relatively constant over the 5 days in vitro (511 ± 66 µm² in 1-day cells and 643 ± 49 µm² in 5-day cells).

The membrane strain-indentation relationship of the modified single-well device was determined for comparison to the original cell-stretching device (Fig. 1C, dashed line). Because equibiaxial cell deformations were to be computed as changes in surface area, membrane strain was converted to change in surface area (Eqs. 4 and 5). The modified device is 50% larger to allow microscopic visualization of the cells; hence differences in membrane deformations between the two devices are due to unscaled dimensions (Table 1). Regardless, the two devices provide nearly identical strain magnitudes, allowing the relationship between cell and membrane deformation to be determined over the full working range of both devices with the microscope-mountable single-well device.

The change in two-dimensional CSA was measured (Eq. 6) as a function of indentation depth for six cells from each of three membranes for 1- and 5-day cells (Fig. 5). In general, the cells exhibit a very consistent response to equibiaxial membrane deformation, with a significant difference between 1- and 5-day cells at only...
one of the nine indentation levels examined and the only significant difference between cell and membrane deformations occurring in 5-day cells near the maximum indentation level.

Cell viability results. Equibiaxial strain-induced injury in 5-day cells was dramatically dependent on the degree of cell spreading (Fig. 6B), which depended on the original seeding density of the wells (Fig. 6A). The percentage of cells dead after a 37% cyclical change in surface area for 1 h increased from ~9% in cells that cover an average area of ~600 µm² to nearly 100% in cells that cover >1,800 µm².

To determine changes in cell response due to time in culture alone, independent of changes in cell spreading, all subsequent studies were carried out in cells seeded at 1 × 10⁶ cells/cm². At this seeding density, the surface area covered by ATII cells increased <25% between 1 and 5 days. Cells maintained in culture for 5 days were significantly less injured by identical deformation protocols than cells maintained for 1 day (Figs. 4 and 7). Injury increased significantly with deformation magnitude regardless of the duration of deformation or the time elapsed after seeding (Fig. 7). This increase occurred over the entire deformation range tested in 1-day cells but was highly nonlinear in 5-day cells in which injury increased markedly when deformations exceeded 37%ΔSA in both the cyclic and single-stretch cases. A single deformation was significantly less injurious than repeated cycling in all cases except for 12%ΔSA (Fig. 7). Fluid flow over the cell culture surface did not result in significant changes relative to control wells (3.0 ± 0.6 and 3.4 ± 2.8% dead, respectively).

DISCUSSION

Mechanical deformation plays an important role in regulating both normal and pathological responses of the lung (39). Although cell culture studies provide an excellent opportunity to examine the effect of well-defined deformations on individual cell types, the in vitro systems used previously to examine alveolar epithelial responses to deformation provide nonuniform strain fields in which cellular deformation is not always known.

The device detailed in this communication provides uniform and equibiaxial changes in MSA of up to ~50% (Figs. 1 and 3), which is adequate to simulate the changes in alveolar epithelial basement MSA that occur on inflation up to and beyond total lung capacity (Fig. 2). Although in vivo cell deformations are most likely heterogeneous, the equibiaxial strain field provided by this device allows a large population of cells to be subjected to a uniform, quantifiable strain, providing a simple correlation between cell deformation and response.

ATII cells in primary culture conditions were found to attach and spread on fibronectin-coated Silastic.
membranes and remained attached during all deformations provided by the device. By restricting cell attachment to the central portion of the cell culture substratum, all cells were subjected to identical substratum strains in all directions during stretching, eliminating uncertainty about the deformation magnitude associated with changes in cell physiology. Any heterogeneity in the cellular response to mechanical stimuli is thus due solely to individual cellular properties such as cell-matrix and cell-cell attachments, cytoskeletal structure, and cell morphology.

We examined cellular injury at 1 and 5 days after seeding because of the dramatic changes in cell morphology and phenotype that occur during this time period. Primary cultured ATII cells (seeded at \( \sim 0.5 - 1.5 \times 10^6 \) cells/cm²) gradually lose many of the specific features of the ATII cell phenotype while simultaneously acquiring certain features of alveolar type I (ATI) cells in vivo. The most obvious morphological change with time in culture is cell spreading, which results in attenuated cytoplasmic extensions and protruding nuclei, similar to ATI cells in vivo (2). Alveolar epithelial cells maintained in primary culture gradually change their pattern of cell type-specific lectin binding (12) and develop immunoreactivity with ATI-specific monoclonal antibodies (9, 13). Over time, the cells lose their lamellar bodies and their ability to express surfactant proteins (10), both characteristics of ATII cells, and increase their expression of intercellular adhesion molecule-1 (5), a trait associated with ATI cells in vivo. Finally, there is evidence that the actin and intermediate filament cytoskeleton is modified by alveolar epithelial spreading in culture (33, 45). All of these changes occur over the first 4–6 days after cell seeding, leading us to choose 5-day cells as representative of this modified phenotype. One-day cells were examined at 24 h, the first time point at which ATII cells were sufficiently attached and spread to be reproducibly deformed. Because the alveolar epithelial phenotype is partially controlled by cell-matrix interactions (43), it must be emphasized that our results are specific to the limited in vitro condition we investigated, primary cultured ATII cells on fibronectin-coated Silastic membranes.

Cells at both 1 and 5 days after seeding experienced reproducible deformations nearly identical to those of the cell culture substratum (Fig. 5). Although previous studies have often neglected this issue, those that have measured cellular strains have reported varying relationships between substratum and cellular deformations. In a study of endothelial cells with biaxial deformation, Winston et al. (52) found cell elongation to be \( \sim 60\% \) of membrane elongation. In a study of primary cultured ATII cells, Wirtz and Dobbs (53) found the ratio of changes in CSA to those in MSA to be \( 0.40 \pm 0.11 \) for fibronectin-coated Sylgard membranes and \( 0.75 \pm 0.36 \) for fibronectin-coated Silastic sheeting. In our studies, there was very little difference between cell and substratum deformations and almost no difference between 1- and 5-day cells (Fig. 5). Although Wirtz and Dobbs speculated that fibronectin attachments might be broken during stretching or that the matrix may slide on the membrane in their preparation, our results suggest an intimate association between the cells and the membrane-bound fibronectin.

To examine deformation-induced injury in alveolar epithelial cells, we selected a commercially available fluorescence cell viability kit (LIVE/DEAD, Molecular Probes). In preliminary experiments, we found this assay to be more sensitive and reproducible than the lactate dehydrogenase (LDH) assay, another commonly used marker of cell membrane breakdown. By visualizing the cells, we were able to examine the homogeneity...
of the response to deformation and confirmed that the cell-stretching device created a uniform deformation field as evidenced by the random and well-distributed pattern of cells stained with ethidium homodimer-1 (Fig. 4). Although fields were selected for analysis randomly, occasional fields that contained cellular domes filled with fluid were excluded. These fluid-filled domes, which indicate tight junction formation and active transport (32), were not photographed because of the unknown strain field in the cells. Rare ATII cells that were attached but maintained a rounded morphology at 1 day were not injured in any protocol and were excluded from counting because of the likelihood that they experienced little or none of the substratum deformation.

In preliminary work, no difference was found between adding the fluorescent dyes before or after the stretching protocol; hence all experiments were carried out with the dyes added before stretching, with microscopic visualization carried out 1 h after the beginning of the stretching protocol. No cells were found to be double stained with both fluorescent markers, indicating that the cells were not able to recover from the membrane disruption that allows ethidium homodimer-1 to enter the cells (22). To verify this over a longer period, a limited number of wells were followed for 24 h after the conclusion of stretching, with new calcein AM added at each examination time. At no time did the cells marked with ethidium homodimer-1 recover their ability to concentrate calcein AM, thus demonstrating that cells were unable to restore membrane function. Therefore, in our preparation, ethidium homodimer-1 stained cells were irreversibly damaged and were not transiently injured as reported elsewhere (22).

ATII cells do not proliferate appreciably in culture (49), so that the mean cell size at 5 days is dependent on the original seeding density (Fig. 6). Therefore, before comparing deformation-induced injury in cells from different days after seeding, it was necessary to examine the effect of seeding density. Our results demonstrate a dramatic dependence of cellular injury on seeding density and mean cell size in 5-day cells (1-day cell size was independent of seeding density). The largest cells we examined covered a surface area approximately equivalent to that covered by a typical ATII cell in rats and humans (~5,000 µm²; Ref. 8). No cells at this density were viable after 1 h of cyclic changes in surface area, equivalent to inflation to total lung capacity, 37%SA (Fig. 6). Approximately 90% of cells, which cover a mean surface area of ~600 µm², remained viable after the identical equibiaxial deformation protocol. This latter cell size is only ~25% larger than the size of cells at 1 day after seeding; thus cells seeded at high density allowed us to examine changes in equibiaxial deformation-induced cell injury, with time after seeding independent of cell spreading.

In experiments with cells seeded at high density (1 × 10⁶ cells/cm²), the most striking finding was the dramatic decrease in equibiaxial deformation-induced injury at 5 days after seeding compared with cells stretched at 1 day after seeding (Fig. 7). The phenotypic and morphological alterations detailed above suggest that ATII cells maintained in culture for 5 days, although not covering the same area as ATII cells in vivo, share many of the characteristics of ATII cells (34). The development of tight junctions and active transport, as evidenced here by fluid-filled dome formation in high-density cultures at 5 days, also indicates that cell monolayers have properties similar to those of the intact epithelium (32). Finally, the development of resistance to equibiaxial deformation-induced injury during 5 days in culture suggests that these cells may be a reasonable representative of the alveolar blood-gas barrier. In the intact lung, this barrier is composed mostly of ATII cells (covering 93–97% of the alveolar surface), providing the majority of the resistance to fluid passage from the vasculature to the air space and allowing for efficient and rapid gas diffusion (7). In the absence of a primary culture of ATII cells, 5-day cells offer an in vitro substitute for examining changes in alveolar epithelial integrity with deformation (34).

Equibiaxial deformation-induced injury in 5-day cells was found to be a highly nonlinear function of deformation magnitude, with negligible cell death at single deformations < 50%SA and cyclic deformations < 25–37%SA (Fig. 7). It is instructive to consider these limits in terms of the lung volumes to which they correspond (Fig. 2). In isolated rat lungs, the alveolar epithelial basement MSA increases ~25 and 37% on inflation, from residual volume to 80 and 100% of total lung capacity (~12 and 25 cmH₂O transpulmonary pressure, respectively; unpublished data). A 50%SA is greater than that normally achieved in a homogeneously inflated rat lung and was selected to be representative of regional overinflation in diseased lungs exposed to large volumes.

Damage to the alveolar epithelial barrier due to deformation most likely occurs as a continuum of responses, from mild functional impairment to gross structural failure. The outcome measure of the study presented here, breakdown of cell membrane integrity, is at the extreme end of this continuum. More subtle changes in barrier properties may include increased leakiness of the tight junctions connecting alveolar epithelial cells (38, 50) or changes in active transport across the alveolar epithelial barrier (46). Reversible structural and functional changes in blood-gas barrier integrity have been reported during some in situ and in vivo lung inflation studies (17, 21). The results obtained from our fluorescent cell viability assay appear to represent an irreversible breakdown of cell membrane integrity. Egan and colleagues (18–20) reported gradual changes in epithelial equivalent pore dimension during static inflation, followed by irreversible injury at high lung volumes. Our in vitro findings support the hypothesis that, at high lung volumes, the alveolar epithelium undergoes irreversible cell damage, leading to failure of the alveolar epithelial barrier. Although measurement of paracellular permeability changes with deformation are beyond the scope of the present investigation, a more sophisticated understanding of acute and
chronic responses of the alveolar epithelium to deformation will be gained by examining these and other deformation-related changes in cell physiology.

Although it remains to be determined what role in vitro deformation plays in altering paracellular permeability (i.e., changes in tight junction integrity), the cell culture results presented here suggest that the viability of the alveolar epithelium should not be adversely affected by a single inflation to total lung capacity or greater or by repetitive inflation to volumes close to total lung capacity. These results make intuitive sense, and there are some data in the literature to support this interpretation. Animal ventilator studies indicate with regularity that the blood-gas barrier begins to break down, as indicated by edema formation, histopathological changes, and impaired lung mechanics, only when peak transpulmonary pressures exceed 25–30 cmH₂O (6, 14, 37, 47, 48). In rabbit lungs fixed in situ after exposure to high transmural pressure, epithelial “breaks” were markedly increased when inflation pressure was elevated from 5 to 20 cmH₂O (23). None of the epithelial breaks occurred in the vicinity of an intercellular junction, suggesting that junctional strength may be greater than the failure limit of the epithelial cell and that cell viability may be killed in preserving junctional integrity. All of these results suggest that the alveolar epithelial barrier remains intact when inflations are limited to below total lung capacity in uniformly inflated lungs, which is in agreement with our finding that, in 5-day cells, equibiaxial deformation-induced injury increases rapidly only when deformations exceed those associated with inflation to total lung capacity (37%ΔSA).

Although ATII cells, which are incapable of proliferation, serve as a barrier between the air space and the vasculature, ATII cells act as the progenitor for repair of the alveolar epithelium in vivo (31). The vulnerability of primary cultured ATII cells at 1 day after seeding to equibiaxial deformation-induced injury reported here (Fig. 7) suggests that ATII cells, which are predominantly located in alveolar corners in vivo (4), may be protected from large deformations that could result in injury. Alternatively, although 1-day cells were examined at 24 h to ensure that most of the ATII phenotype was preserved, there is reason to believe that this preparation may result in ATII cells in the beginning stages of repair rather than in their quiescent state. At 1 day, the ATII cells covered a significantly larger surface area (~500 μm²) than in vivo ATII cells (~150 μm²; Ref. 8), indicating a much greater degree of cell spreading and a different cell-substratum interface than found in vivo. In addition, the use of fibronectin, which is found in elevated concentrations in the alveolar epithelial basement membrane during repair of the adult lung, accentuates cell spreading and may be involved in signaling the cell to enter a repair phase (45). This raises the possibility that the ATII cells that remain rounded at 1 day, which were uniformly uninjured and were excluded from analysis, may actually be more representative of in vivo ATII cells. Finally, it must be mentioned that cell deformations were selected based on the average changes in epithelial basement MSA with lung inflation (unpublished data). Because only 3–7% of the alveolar surface is covered by ATII cells in vivo (8), it is possible that ATII cell deformations in vivo are considerably smaller than those experienced by the alveolar epithelium on average.

The only other report on changes in alveolar epithelial cell viability with known cell deformations comes from Wirtz and Dobbs (53), who examined deformation-induced calcium mobilization and phosphatidylcholine secretion in ATII cells 20–24 h after seeding. They measured LDH released during stretching and expressed the results as a percentage of the total cellular LDH content. LDH is an alternative marker of the breakdown of cell membrane integrity, and the percent LDH released is analogous to our measurement of the percentage of dead cells. They discarded occasional control and stretched wells in which LDH release exceeded 1%. However, they were able to subject cells to single deformations that resulted in a range of changes in CSA up to 25% without >1% LDH release. This is in marked contrast to our single-stretch results in 24-h cells, where we found 16.3 ± 9.2% dead cells at 25%ΔSA. There are several possible explanations for this discrepancy. Although both assays of cell viability measure membrane permeability, the assays utilize molecules of vastly different molecular masses (LDH ~180 kDa and ethidium homodimer-1 ~1 kDa) to demonstrate cell death. Also likely to contribute to the discrepancy is the nonhomogeneous nature of the deformation field in the device used by Wirtz and Dobbs (53; see also Refs. 25, 51). Although individual cells were analyzed for calcium mobilization and changes in CSA, the LDH assay gave a result averaged over the entire cell culture surface, portions of which most likely experienced deformations significantly smaller than those for which measurements of cell dimensions were taken (25, 51). A final possibility is that cell-substratum attachment in the Wirtz and Dobbs (53) study, which resulted in vastly different cell and substratum deformations, may have allowed failure of cell attachments rather than subjecting the cells to lethal deformations.

The reduced injury reported here in response to a single stretch, in contrast to repetitive cycling (Fig. 7), indicates that cellular injury is cumulative for at least some of the cells at both 1 and 5 days. It also suggests that, in this cell culture preparation, some cells within a well are more predisposed to injury than others because of a variety of undetermined cell-specific features. In the intact lung, these cells would almost certainly be eliminated by their inability to withstand normal deformations and would be replaced by cells better suited to the mechanical environment. Future studies with in vitro preparations could address this possibility by preconditioning the wells with repetitive small deformations and allowing for repair time to see whether a more mechanically robust monolayer would result.

Our results indicate that equibiaxial deformation-induced injury in alveolar epithelial cells is a function not only of deformation magnitude and duration but...
also of the seeding density, degree of cell spreading, and phenotypic state of the cells. These results, coupled with those from neural (22) and smooth muscle preparations (3), indicate that cellular tolerances of deformation may vary widely from tissue to tissue, within morphologically distinct cells from identical sources, and even among cells with nearly identical morphology. The various tissues of the body are exposed to a wide variety of deformation fields, and their ability to withstand and remodel in response to stress and strain are fundamental properties with important implications in a variety of fields. More investigation into the cell- and tissue-specific determinants of deformation-induced injury is needed to understand the biomechanical tolerances of tissues at the cellular and molecular level.

The major goal of this work was to develop an in vitro model capable of subjecting alveolar epithelial cells to deformations that mimic those of the intact lung. This in vitro preparation allows the investigation of cellular responses to well-defined and homogeneous deformations in isolation from changes in blood gases, surfactant activity, and inflammatory responses that can accompany in vivo or isolated lung studies. Such a model is useful because it eliminates these complicating variables and can be used to study a range of functional responses to deformation at the cellular and molecular levels.

In summary, we have demonstrated that the cell-stretching device described here provides a uniform, equibiaxial deformation field that is unchanged by 1 h of continuous cycling. Alveolar epithelial cells cultured in custom-made polysulfone wells on fibronectin-coated Silastic membranes deform in a manner identical to the substrate in both 1- and 5-day cells. By providing predictable, uniform strains to a population of alveolar epithelial cells, this device allows more quantifiable investigations of the response of the alveolar epithelium to deformation and will further our understanding of the importance of mechanical stimuli in regulating lung structure and function. As a first step in this process, we examined injury caused by changes in CSA. Equibiaxial deformation-induced injury was found to increase with deformation magnitude and duration and to decrease with seeding density and time after seeding. Our results suggest that ATII cells are protected from large deformations in vivo by their preferential location in alveolar corners and that 5-day cells behave in a manner similar to ATI cells in vivo, with injury increasing rapidly as deformations exceed physiological values. These findings indicate that deformation-induced injury may be a critical factor in the development of lung injury during mechanical ventilation with high end-inspiratory lung volumes.

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