Validation of a new live cell strain system: characterization of plasma membrane stress failure

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Stroetz, Randolph W., Nicholas E. Vlahakis, Bruce J. Walters, Mark A. Schroeder, and Rolf D. Hubmayr. Validation of a new live cell strain system: characterization of plasma membrane stress failure. J Appl Physiol 90: 2361–2370, 2001.—Motivated by our interest in lung deformation injury, we report on the validation of a new live cell strain system. We showed that the system maintains a cell culture environment equivalent to that provided by conventional incubators and that its strain output was uniform and reproducible. With this system, we defined cell deformation dose (i.e., membrane strain amplitude)-cell injury response relationships in alveolar epithelial cultures and studied the effects of temperature on them. Deformation injury occurred in the form of reversible, nonlethal plasma membrane stress failure events and was quantified as the fraction of cells with uptake and retention of fluorescein-labeled dextran (FITC-Dx). The undeformed control population showed virtually no FITC-Dx uptake at any temperature, which was also true for cells strained by 3%. However, when the membrane strain was increased to 18%, ~5% of cells experienced deformation injury at a temperature of 37°C. Moreover, at that strain, a reduction in temperature to 4°C resulted in a threefold increase in the number of cells with plasma membrane breaks (from 4.8 to 15.9%; P < 0.05). Cooling of cells to 4°C also lowered the strain threshold at which deformation injury was first seen. That is, at a 9% substratum strain, cooling to 4°C resulted in a 10-fold increase in the number of cells with FITC-Dx staining (0.7 vs. 7.5%, P < 0.05). At that temperature, A549 cells offered a 50% higher resistance to shape change (magnetic twisting cytometry measurements) than at 37°C. We conclude that the strain-injury threshold of A549 cells is reduced at low temperatures, and we consider temperature effects on plasma-membrane fluidity, cytoskeletal stiffness, and lipid trafficking as responsible mechanisms.

biomechanics; cell physiology; alveolar epithelial cells; ventilator-induced lung injury

MECHANICAL VENTILATION is the cornerstone of treatment for patients with the acute respiratory distress syndrome. However, ventilator settings required to sustain life can at times injure the lungs by overdistending them (9, 42). To date, two studies have shown that mechanical ventilation with large tidal volumes (12 ml/kg) is associated with greater patient mortality than mechanical ventilation with low tidal volumes (6 ml/kg) (2, 33). Similar observations had been made in animal models of ventilator-induced lung injury, in which lung damage can be prevented or reversed by reducing tidal volume (9, 23, 34, 41). Damage is characterized by edema, hemorrhage, the presence of inflammatory cells, and changes in the ultrastructure of alveolar lining cells (5, 9, 16). Such changes consist typically of epithelial and endothelial membrane blebs and breaks and are presumably the sequelae of intermittent plasma membrane-wounding events (21, 32, 37). However, the micromechanics of the alveolar space and the deformation of its cellular constituents during breathing are incompletely understood (22, 45). As lung volume increases from functional residual capacity, the alveolar septa are thought to mostly unfold, accompanied by only minor deformations of their cellular constituents (4, 22, 36). However, at high lung volumes, the septa must withstand a stretch to accommodate the volume-dependent change in alveolar surface area. Therefore, both basement membrane and its adherent cells are placed under a potentially injurious stress (5).

Because classic morphometric techniques require tissue dehydration and fixation (3), different in vitro devices have been developed to simulate mechanical ventilation and to study the relationship between basement membrane strain and deformation responses in living cells (19, 24, 38). Such devices usually incorporate a deformable elastic membrane (in vitro equivalent of the alveolar basement membrane) capable of supporting lung cell growth, and some make provisions for cell imaging under more or less physiological environmental conditions. Most devices, however, have shortcomings, including nonuniform and/or poorly characterized membrane strain fields, the inability to precisely and independently control strain rate and...
strain amplitude, and restricted imaging capabilities and/or a lack of environmental controls essential for live cell microscopy (6, 19).

In this communication, we present our validation of a prototype cell deformation system that overcomes these limitations. By placing optical position sensors in a closed feedback loop with linear actuators, membrane strains are continuously monitored and adjusted to produce precise strain rates and amplitudes. Furthermore, cells were maintained in an environment equivalent to that of an incubator, thus making long-term deformation response studies feasible. We used this device to characterize the relationship between substratum deformation and plasma membrane stress failure (wounding) in alveolar epithelial cells. With a different system, we had previously shown that large deformations (peak membrane strain ~25%) cause plasma membrane breaks in these cells (37) but because of device limitations could not further define the “deformation dose-cell wounding response” relationship. We now show that >98% of A549 cells (a human alveolar epithelial cell line) withstand a single 90-s stretch of 9% (equivalent to a basement membrane area increase of ~18%) without undergoing plasma membrane injury, but that larger deformations greatly increase their probability of plasma membrane stress failure. Finally, we show that temperature has important effects on the mechanical properties of cells and on their probability of experiencing plasma membrane stress failure. The biological implications of these observations are discussed.

METHODS

Design of Strain Device

Conceptually, the straining system is similar to others presented previously (14, 17, 28, 35), and its basic principle is commonly used in vacuum, hydraulic, and pneumatic devices found in industry. Two concentric cylinders and an elastic membrane function as diaphragm and piston. An elastic membrane (i.e., the diaphragm) covers one end of the large cylinder much like the head of a drum. When the large cylinder descends over the smaller inner cylinder, its membrane (the diaphragm) gets radially stretched (strained) in direct proportion to the vertical displacement. This is illustrated schematically in the relaxed (Fig. 1A) and strained (Fig. 1B) configurations. The diaphragm appears as a circular fixture that clamps the elastic membrane. The membrane becomes strained (Fig. 1B) over the piston when vertical displacement of the diaphragm occurs via two stepper motor-driven rack and pinion gear mechanisms. The portion of the diaphragm residing over the piston remains in the same plane as mechanical straining occurs, and the motion hardware is located to the sides of the mechanism. This arrangement allows unobscured microscopic imaging over the radially strained portion of the membrane. In the actual prototype, the diaphragm and piston are inserted as subcomponents or modules into upper (vertically movable) and lower (vertically stationary) rigid aluminum frames. This modular arrangement allows flexibility in the choice of subcomponents, facilitating the development of new prototype biomembranes and cell culture systems concurrent with real-time imaging.

Mechanically, vertical displacement of the diaphragm assembly is accomplished via two linear actuators (stepper motor-driven ball bearing-loaded leadscrows, Ultra-Motion, Mattituck, NY). The linear actuators are driven by integrated microstepping controllers (IMS model 483IE2, Intelligent Motion Systems, Marlborough, CT). Optical encoders that continuously output motor position are incorporated in a closed feedback loop, indexing initial motor position and automatically correcting errors. User control of the system is provided by in-house-developed software (Labview, National Instruments, Austin, TX), a PC (Pionex, Somerset, NJ), and serial communication with the stepper motor drive systems. The user interface provides control in a similar fashion to mechanical ventilation, allowing manipulation of the cycling frequency, duty cycle, strain amplitude (analogous to tidal volume), strain rate (analogous to mean inspiratory flow), and a prestrain (analogous to positive end-expiratory pressure).

Design of Cell Growth Chamber

The prototype’s upper frame houses the biologically active diaphragm module and is fitted with an opaque plastic enclosure that functions as an incubator (Fig. 2). Gas is circulated through the plastic enclosure after being filtered (HME 15–22, Pall Biomedical, Fajardo, PR), heated, and humidified by a humidification system commonly used with mechanical ventilation (Bird Products, Palm Springs, CA). This system uses a remote thermistor temperature probe in a closed feedback control system with the heating element to provide precise temperature control remotely to the heating source. Air and CO2 flows at 5.0 and 0.5 l/min, respectively, are blended with calibrated flowmeters (Ohmeda, Columbia, MD, and Dwyer Instruments, Michigan City, IN) and introduced to the heater/humidifier. At these flows, CO2 tension measured with an infrared capnostat (Novametrix model 1260, Wallingford, CT) is 37 mmHg. The straining system is housed within a bio-hood (AirClean 600, Air Clean Systems, Raleigh NC) that has been extensively modified to allow
Validation of Strain Performance

The prototype’s lower frame was fitted with six Teflon pistons of 25 mm OD and 22 mm ID, with 1-mm radius between the top and side surfaces. The piston surfaces that contact the membrane were coated with a lubricant (Braycote 804, Castrol, Commerce, CA) to reduce friction. A six-well culture plate (Bioflex, Flexcell International, McKeesport, PA) was inserted into the upper frame. The relationship between displacement amplitude and membrane strain in a piston-diaphragm system is well established (6, 14, 28), and our validation procedure is essentially the same as that described by Tschumperlin and Margulies (35). In short, real-time motion of five fiducial markers on the membrane’s surface was recorded with a charge-coupled device video camera (Sony CCD F40, Tokyo, Japan) during programmed displacements of the linear actuator (Fig. 3). A video frame grabber and software developed in our laboratory were utilized to digitize and compute the distance between each pair of fiducial markers over the full range of actuator positions (13, 20). Strain was defined as the fractional length change of the line elements between marker pairs. Analysis of variance was used to test whether the orientation of individual marker pairs had an effect on the strain-actuator position relationship. This analysis served to test whether the strain field was uniform in the radial direction. To describe the dependence of membrane strain on actuator position under static conditions (actuator held in position for several seconds), the mean data from all pairs were fit to a fourth-order polynomial. The displacement was defined in a similar manner.

Characterization of Cell Culture Viability

The following indexes of cell viability and function were compared between culture environments: cell number, pH (using a Beckman model 10 pH meter; Beckman Instruments, Fullerton, CA), and osmolality of the supernatant; proportion of cells with trypan blue positive cells from either the incubator or strain device (six separate experiments, i.e., 30 wells).

Trypan blue exclusion assay. Cells were trypsinized, and 0.5 ml of 0.4% trypan blue (Sigma Chemical) was added to a 0.5-ml cell suspension and allowed to stand for 5–7 min. A 100-μm-deep hemacytometer (Hauser Scientific, Horsham, PA) was used to count both the total number and number of trypan blue positive cells from either the incubator or strain device (six separate experiments, i.e., 30 wells).

51Cr release assay. Cells were incubated for 2 h with 20 μl of 51Cr (1 μCi/μl) added to the growth medium (six separate experiments). The 51Cr-containing medium was then discarded, the cells were washed, and the medium was replaced with unlabeled medium. The amount of 51Cr released into the supernatant over the ensuing 2 h was measured using a gamma counter (Ludlum Measurements, Sweetwater, TX) and expressed as a fraction of the radioactivity in the cell lysate (1% Triton; Sigma Chemical).

Flow cytometry and PI labeling. In three separate experiments (36 wells in each group), the cells were trypsinized and diluted into ice-cold phosphate-buffered solution. After 7.5 μl/ml of PI was added, the cells were maintained on ice until flow analysis was performed. Cells were analyzed on a FACSCalibur (Becton-Dickinson, San Jose, CA) flow cytometer. Argon ion laser light (λ = 488 nm) was used to excite PI, and emission fluorescence was detected with a 585 ± 21-nm filter. Forward-angle scatter, side scatter, and geometric mean fluorescent intensities were recorded from 10,000–20,000 cells and analyzed using Cell Quest flow cytometry software (Becton-Dickinson). Only cells above a forward-angle scatter threshold, distinguishing cells that could be evaluated, were analyzed.

Characterization of Radial Cell Growth Rate

Only in areas of the membrane that overlie the piston in the displaced state is substratum strain uniform and defined

Flexible, collagen I-impregnated silicoelastic membrane with a surface area of 9.6 cm². Ham’s F-12K 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 a growth medium. A549 cells were passaged and seeded at a density of 20,800 cells/cm² (200,000 cells/well) 48 h before each experiment.

Cell Culture

Human A549 cells (American Type Culture Collection, Rockville, MD) were grown on six-well culture plates (Bioflex, Flexcell International) in either a standard cell culture incubator (model 3226, Forma Scientific, Marietta, OH) or the strain device. The base of these culture plates consists of a...
by actuator position. For this reason, it is important to restrict initial cell attachment to the center portion of the membrane and also to ascertain that the subsequent radial growth remains confined to the part of the membrane with a uniform strain field. The area over which this condition is met is a circle with the radius $r_{\text{crit25}}$. This critical radius varies with actuator displacement and is 9.2 mm at static settings of 25%. Cells were seeded inside a Teflon ring with an inner radius of 7.5 mm. After 48 h, the Teflon ring was removed, and cell growth and spreading were measured over the ensuing 70 h. The distance of the most peripheral cells relative to $r_{\text{crit25}}$ was measured at four points (0°, 90°, 180°, and 270°) in each of six wells at seven time points over a 72-h period by use of a calibrated scale within the imaging path of a light microscope.

**Determination of Deformation-Induced Plasma Membrane Breaks**

To determine whether cellular deformation was associated with breaks in the plasma membrane, cells were incubated with fluorescein-labeled dextran (FITC-Dx, Fluka, Milwaukee, WI; molecular mass = 70 kDa) for 10 min and then deformed (21). A single stretch of 1.5 min was applied to cells maintained at 37°C, room temperature (21°C), or 4°C. Cells with homogenous intracellular distribution of FITC-Dx were counted in five low-power (×40 water immersion objective lens, numerical aperture = 0.75; Carl Zeiss, Thornwood, NY) microscope fields in each culture well for each experiment and were tabulated as a percentage of the total number of cells counted (between 200 and 300 cells in total). Four separate experiments were performed for each combination of strain amplitude and temperature. Within each six-pack of culture wells, three wells were strained and three were not strained, serving as a control cell population.

**Magnetic Twisting Cytometry**

Magnetic twisting cytometry (MTC) was used to measure the temperature dependence of apparent cell stiffness (12, 40). Ferromagnetic ($Fe_3O_4$) microbeads (4.5-μm diameter) were coated with a synthetic RGD peptide (RGD Peptide, Telios Pharmaceuticals, San Diego, CA) at a concentration of 50 μg/ml and allowed to associate with the cells (1-2 beads/cell) for 20 min. Cell wells were then placed into the magnetic twisting cytometer at an initial temperature of either 37 or 4°C, chosen in random order. A 10-μs 1,000-G homogeneous magnetic pulse was then applied to magnetize the beads in the horizontal direction. A fluxgate magnetometer (Poerster, Reutlingen, Germany) was used to measure the remnant magnetic field of the beads in the horizontal direction. A magnetic “twisting” field (<80 G), H, was applied in the vertical direction to twist the beads upward. Cell mechanical properties were derived from twisting torque and angular bead rotation, as previously described (12, 40).

**Statistical Analysis**

All measurements are presented as means ± SD. Statistical comparisons between experimental conditions were made using the Student t-test for paired or unpaired observations, as appropriate. Statistical significance was assumed at $P < 0.05$ with respect to a two-tailed probability distribution. Analysis of variance was used to determine membrane strain uniformity and to test for temperature effects on the cell strain-plasma membrane wounding relationships.

**RESULTS**

**Characterization of the Actuator Displacement-Membrane Strain Relationship**

Figure 4 shows several actuator displacement-membrane strain relationships measured over the operating range (100% displacement) of the device under both static and dynamic conditions. The dashed line through open circles is the fourth-order polynomial fit to the static data. Accordingly, a maximal actuator displacement, when held for several seconds, generated a 28% membrane strain. The actuator returned to the default position between each displacement step, and individual strains were referenced to the preceding membrane state (i.e., the 0 strain position). In all cases, the variability in strain estimates between different marker pairs was <1%. The variance about each strain estimate is quite small and reflects local differences in membrane elastance attributable to variations in membrane thickness and prestress as well as tracking measurement errors. The distance between individual marker pairs at the default position varied by less than ±2%. ANOVA revealed no significant differences in strains computed from marker pairs with different orientations ($P > 0.3$), indicating relative uniformity in strain field over the piston-opposed membrane.

During dynamic testing, the strains at any given actuator position were less than in the static case. This is not surprising given frictional losses between membrane and piston. Interestingly, these losses were more or less frequency independent, i.e., the differences in membrane strain between static and dynamic runs did not vary with actuator displacement rate. The shapes of the curves reflect membrane stiction (during the initial 10% of actuator displacement) and membrane

Fig. 4. Plot of membrane strain as a function of actuator displacement. Data are means of strain estimates of all dot pair combinations shown in Fig. 3. Actuator displacement-membrane strain relationships measured over the operating range (100% displacement) of the device are shown under both static and dynamic conditions. The dashed line through open circles is a 4th-order polynomial fit to the static data (equation shown). The membrane strain at any actuator position is less under dynamic conditions. See text for more details.
creep at the end of the actuator downstroke (between 80 and 85% in this example). The four actuator displacement rates tested produced average membrane strain rates of 41, 21, 7, and 2% per second.

Cells are Viable and Function the Same as They Would in a Conventional Incubator

Several measures of cell viability were compared between cells grown in the strain system and cells grown in a conventional incubator (Fig. 5). In six separate experiments, each covering a time span of 48 h, we found no significant difference in the cell growth rates (cell number $1.63 \pm 0.45$ vs. $1.60 \pm 0.49 \times 10^6$, $P > 0.6$; Fig. 5A) or the percentage of cells that took up trypan blue ($5.4 \pm 1.9$ vs. $6.4 \pm 3.6\%$, $P > 0.3$; Fig. 5B, left). Similarly, the pH of the supernatants of both cell populations measured 48 h after incubation on six separate occasions in six wells were each similar, i.e., $7.37 \pm 0.07$ and $7.34 \pm 0.05$ ($P > 0.4$). In a single comparison between 12 wells each, the osmolality of the supernatants was $342 \pm 4.6$ mosmol/kg in the strain system compared with $338 \pm 1$ mosmol/kg in the incubator ($P < 0.01$).

In three separate experiments on six wells each, cells maintained either in the prototype cell strain system or the incubator showed no significant difference in PI uptake ($4.8 \pm 0.5$ vs. $5.4 \pm 1.4\%$, $P > 0.5$; Fig. 5B, right). A similar comparison of the 2-h $^{51}$Cr release data likewise revealed comparable viability results (Fig. 5C). However, a paired analysis of the data from six separate experiments shows a small, yet statistically significant trend in favor of the conventional culture environment ($^{51}$Cr release: $11.3 \pm 2.4$ vs. $9.9 \pm 1.7\%$, $P < 0.05$).

Cell Growth Relative to the Membrane Area With Uniform Strain Characteristics

To ensure that A549 cells adhered only to those portions of the membrane with defined uniform strain characteristics, the monolayer was imaged, and its radial growth was defined relative to $r_{crit25}$. Recall that $r_{crit25}$ defines the circular membrane area with uniform strain characteristics at a 25% strain setting. Data in Fig. 6 from two representative experiments (4 measurements per time point per well, 6 wells per experiment) show that, over a 70-h period, none of the A549 cells spread or crawled beyond $r_{crit25}$.

Plasma Membrane Injury is Strain Amplitude, Strain Rate, and Temperature Dependent

Figure 7 shows deformation dose (strain)-plasma membrane injury response (%cells with FITC-Dx uptake) relationships at three temperatures, namely 37, 21, and 4°C at maximal actuator displacement rate settings. The undeformed control population showed virtually no FITC-Dx uptake at any temperature, which was also true for cells strained by 3%. However, when the membrane strain was increased to 18%, ~5% of cells experienced deformation injury at a temperature of 37°C. Moreover, at that strain rate and amplitude, a reduction in temperature to 4°C resulted in a threefold increase in the number of cells with plasma membrane breaks (from 4.8 to 15.9%; $P < 0.05$). Cooling of cells to 4°C also lowered the strain threshold at which deformation injury was first seen. That is, at a 9% substratum strain, cooling to 4°C resulted in a...
7.5-fold increase in the number of cells with FITC-Dx staining (0.7 vs. 7.5%, \( P < 0.05 \)).

Not shown in Fig. 7 are the independent effects of membrane strain rate on FITC-Dx uptake, which were tested in cells maintained at a temperature of 37°C and at strain amplitude settings of 18%. Lowering the strain rate from 41 to 21%/s resulted in a 50% reduction in the number of FITC-Dx-positive cells. Strain rates of 2 and 7%/s produced no plasma membrane injury under these experimental conditions.

**Cooling Increases A549 Cell Stiffness**

Figure 8 shows the individual and mean effects of temperature on A549 cell stiffness. The 12 paired observations reflect stiffness measurements from six wells at two applied stress levels (corresponding to 40-G and 80-G twisting fields). Cooling caused a large and consistent increase in apparent cell stiffness, raising it from 141 to 210 dyn/cm\(^2\) at the lower stress \( P < 0.05 \) and from 162 to 274 dyn/cm\(^2\) at the higher stress \( P < 0.05 \).

**DISCUSSION**

Motivated by our interest in lung deformation injury, we have introduced and validated a prototype live cell strain system. We have shown that the system maintains a cell culture environment equivalent to that provided by conventional incubators and that the strain output is accurate and reproducible within the resolution limits of our bench validation instruments. The system allowed us to define cell deformation dose (i.e., membrane strain amplitude)-cell injury response relationships in alveolar epithelial cultures and to study the effects of temperature on them. Consequently, we have demonstrated that the strain-injury threshold of A549 cells is reduced at low temperatures, an observation that is consistent with temperature effects on plasma-membrane fluidity, cytoskeletal stiffness, and deformation-induced lipid trafficking.

**Basic Design and System Validation**

The system deforms the base (membrane) of commercially available culture plates (Bioflex, Flexcell International) by using the diaphragm-piston mechanism depicted in Fig. 1. This mechanism is driven with linear actuators rather than intermittent pressure, because stepper motors offer greater precision and better response times than pneumatic controls. In our hands, driving pressures in excess of 50 kPa (375 mmHg) were required to strain membranes over 2.0-cm-diameter loading posts by >15% in a pneumatic configuration. Commercially available systems with pneumatic controls, therefore, might not generate high enough pressures to mimic the full range of alveolar basement membrane strains that are thought to occur during injurious mechanical ventilation in vivo (4, 22, 26, 36). They also tend to be more susceptible to frictional losses because of the much larger contact area between the membrane and the solid piston underneath. When used without a piston, the application of a transmural pressure produces a spherical deformation with a very nonuniform strain field (6, 28). This greatly complicates the interpretation of deformation dose-cell response data. As predicted, on theoretical grounds, strain was uniform over the part of the mem-

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**Fig. 6.** Radial growth and spreading of the cell monolayer after removal of a mechanical growth barrier. Data shown are means ± SD of 48 microscopic measurements of cell culture boundaries (4 quadrants/well) at 7 time points in 2 separate experiments. \( r_{\text{crit25}} \) is the radius of the circular membrane area with uniform strain characteristics.

**Fig. 7.** Effect of substratum strain amplitude and temperature on alveolar epithelial cell plasma membrane injury. Injury is defined by the cell fraction with cytosolic dextran (FITC-Dx) fluorescence. Data are means ± SD of 4 experiments (3 wells/experiment), each referenced to its own “static,” i.e., 0 strain, control. *\( P < 0.05 \).

**Fig. 8.** Effects of temperature on the apparent stiffness of A549 cells. Measurements were made on 6 wells at 2 external stress settings (corresponding to 40- and 80-G fields). Individual and mean data (±SD) are shown. *\( P < 0.05 \).
brane that remained opposed to the “piston” during actuator displacement. To test this hypothesis, we used a video camera and an operator-interactive tracking system that we had validated previously for other applications (13, 20). The variability in membrane strain estimates for a given actuator displacement was generally <$1% over the operating range of the device.

**Maintenance of a Physiological Culture Environment**

To define physiological responses of live cells to deformation, it is essential to control temperature, humidity, and gas tensions under all experimental conditions. Therefore, we tested the responsible components in an integrated configuration at the bench (data not shown) and subsequently measured several indexes of viability and function in cells maintained for 48 h in either the strain system or a conventional incubator. The osmolality of the supernatant, a test of the humidification system, was 4 mosmol/kg higher in the strain unit than in the incubator. This difference was statistically significant ($P < 0.01$) but likely of negligible biological consequence. Supernatant pH, a measure of the adequacy of CO$_2$ supplementation, was similar in the two culture environments, as were measures of cell growth and membrane integrity. We have no explanation for the small but consistent difference in $2$-h $^{51}$Cr release between strain unit and incubator. Again, this difference falls well below our biological tolerance limits.

**Effects of Deformation on Plasma Membrane Stress Failure**

When alveoli of overventilated lungs are imaged, one is struck by the presence of numerous breaks between and within alveolar epithelial cells (9, 10, 43). This observation suggests that, under conditions of high tidal volume ventilation, the area change of the alveolar basement membrane is too large to be accommodated by adherent epithelial cells. Using the prototype live cell strain system, we sought to simulate this event and establish a relationship between substratum strain and an index of cell wounding. To date, work on plasma membrane “wounding” has focused primarily on stress injuryadaptive responses in heart and skeletal muscles (1, 7, 11, 21). However, it is becoming increasingly clear that plasma membrane wounding occurs in many different cells under both physiological and pathological conditions. Unless membrane defects are very large, cells rapidly repair wounds by site-directed calcium-regulated exocytosis (30). In the context of ventilator-induced lung injury, it is tempting to link proinflammatory signaling responses by deformed alveolar epithelium to wounding-related cytosolic calcium oscillations. The calcium influx associated with an occasional plasma membrane tear might well be sufficient to keep the nuclear transcription factor-$\kappa$B activated without necessarily compromising cell viability (8, 44).

Because in A549 cells Dx uptake via pinocytosis is time dependent (37), we were able to use cytosolic Dx fluorescence as a marker of transient plasma membrane stress failure (21). At body temperature (37°C), such stress failure was observed only after large substratum deformations applied at high rates. This observation raises three important questions: 1) what deformations do alveolar epithelial cells experience during mechanical ventilation at injurious settings; 2) what are the limitations of A549 cells as a culture model of the alveolar space; and 3) what are the mechanisms of force transfer among basement membrane, cytoskeleton (CSK), and plasma membrane?

**Alveolar microstrains 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, the corresponding strains could be as high as 30%. Because in patients with lung injury the amount of recruitable lung is often quite reduced, every ventilator-delivered breath might represent an inspiratory capacity maneuver (27). In recumbent normal dogs, in situ parenchymal strain measurements yielded values as high as 40% during inspiratory capacity inflations (26). In these studies, the parenchymal marker technique (13) was used so that strains could be computed for tissue volumes as small as 1 cm$^3$. It is uncertain, however, that 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 among surface tension, surface area, tissue properties, and capillary pressure on alveolar geometry (4, 22, 36). Although the free alveolar surface area as much as doubles when the lungs are inflated from 40 to 100% total lung capacity, some of the area change reflects unfolding of alveolar septal “pleats” as opposed to extension of elastic cell and tissue elements (4). Consequently, the epithelial basement membrane area might 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 that one might reasonably expect during mechanical ventilation in vivo.

**Limitations of A549 cells as a culture model of the alveolar space.** A549 cells, derived from a transformed alveolar epithelial cell line, have lamellar bodylike structures (18), possess functioning proinflammatory signaling pathways (38), are cheap and easy to maintain in culture, and are, therefore, a popular culture model of the alveolar space. However, these cells do not form polarized monolayers and probably do not communicate with each other through gap junctions. When probed with MTC, A549 cells appear much stiffer than virtually any other cell type studied to date, including primary rat type II alveolar epithelial cells (12). This might explain why Tschumperlin and Margulies (35) found freshly harvested primary rat alveolar type II epithelial cells to be more “deformation injury prone”
than our observations on confluent A549 cells would suggest. No matter which cell culture model one uses, in vitro determined strain injury thresholds need not (and probably do not) reflect the strain sensitivity of alveolar epithelium in a living organism. Nevertheless, ex vivo measurements are extremely helpful in characterizing effects of co-interventions, such as temperature, and in delineating their mechanisms of action.

The mechanics of adherent cells stressed through their attachment sites with the basement membrane. Adherent cells form focal adhesion complexes (FAC) with matrix proteins of the basement membrane and are in a sense “spot welded” to it. These sites are biologically active and are thought to play an important role in mechanosensing and signal transduction (15). When the basement membrane area increases during lung inflation, the distance between FACs must change. The strain on the FACs causes a realignment of cytoskeletal elements and ultimately produces a change in overall cell shape. The resulting nonuniform deformation (the cell flattens) mandates an increase in the apparent cell surface-to-volume ratio (39), which requires cytoskeletal remodeling and/or unfolding of plasma membrane invaginations. Except for bending phenomena, such as interleaflet shear in the lipid bilayer, the unfolding itself leaves the plasma membrane essentially unstressed until the lipid reservoir for unfolding is exhausted (25, 29). However, at that point, plasma membrane stress failure must be imminent because the critical tension of the plasma membrane is thought to be in the neighborhood of 5 dyn/cm². This means that the plasma membrane can sustain strains of only 2–3% (in the plane of the membrane) before it breaks.

Effects of temperature on plasma membrane stress and stress failure. In a previous study, we showed that A549 cells respond to deformation by transporting lipids to the plasma membrane, and we speculated that lipid trafficking represented an important cytoprotective mechanism against lung deformation injury (39). This hypothesis is consistent with findings on human fibroblasts in which plasma membrane tethers can be pulled to ever-increasing lengths so long as they are being pulled slowly (29). This in turn is consistent with the dependence of plasma membrane stress failure on the substratum strain rate that was observed in the present study. Because vesicular and nonvesicular lipid transport is greatly suppressed at low temperatures, we anticipated that cooling would lower the deformation injury threshold of A549 cells as well. This is exactly what was observed (Fig. 7). However, in this context a few comments about cytosolic FITC-Dx as a marker of deformation-related plasma membrane injury are in order. For the large molecule Dx to gain access to the cytosol, it must enter the cell either via pinocytosis or through a plasma membrane wound. In the latter instance, the molecule will be retained by the cell only if its wound heals and Dx gets trapped. Moreover, the size of the membrane wound and the time to resealing determine cytosolic Dx concentration. When we consider the effects of temperature on lipid trafficking and vesicular fusion, cells that were strained at 4°C probably had longer membrane open times than cells that were strained at 37°C. Finally, the percentage of adherent FITC-positive cells underestimates the extent of injury, because cells that lose adherence during strain and those that fail to repair the membrane defect are not taken into account.

Temperature not only modifies lipid transport but also has profound effects on other cell biological functions. Of these, two are of note in the context of our study: cytoskeletal mechanics and membrane fluidity. The MTC measurements of Fig. 8 provide strong evidence that cooling causes a substantial increase in the cells’ resistance to shape change. This holds true not only for A549 cells but indeed for primary rat type II alveolar epithelial cells as well (data not shown). The molecular mechanisms responsible for the low temperature-induced cell stiffening remain unclear, and a discussion of this topic must be preceded by considerations about MTC itself. What does MTC measure? There is ample evidence that MTC probes the CSK, inasmuch as beads that are not associated with the CSK offer little resistance to rotational shear (12, 40). What remains more uncertain, however, is the sensitivity of the probe to local bead coupling phenomena as opposed to global changes in CSK prestress and organization. In a typical MTC experiment, strain recovery is only ~50%, meaning that the beads do not fully “recoil” to their original position after the twisting field is turned off. In other words, cells display plastoelastic behavior. The molecular mechanisms underlying cell plasticity are also incompletely understood but more than likely involve a number of CSK-associated severing, cross-linking, and annealing proteins (31). To the extent to which temperature alters affinity states and chemical reaction rates of these proteins, cold-induced stiffening might be viewed like the rigor state of an ATP-depleted muscle.

Is a stiff cell that is being strained via its FACs more or less likely to undergo plasma membrane injury? The answer to this question requires some idea about how CSK and plasma membrane interact and load each other during a basement membrane deformation. Unfortunately, there are few experimental data to guide us. We know from this and from the work of Tschumperlin and Marquardt (35) that primary type II epithelial cells are more deformable and more injury prone than the relatively stiff A549 cells (35). However, there are undoubtedly additional factors besides CSK stiffness that determine the probability of plasma membrane stress failure. These are the “strength” and composition of the plasma membrane itself, its mechanical coupling with the subcortical CSK, and the size of the membrane reservoir that is recruited during a shape change and keeps membrane tension below injury threshold as the surface area of the cell increases.

Concluding Remarks

We have reported on the testing and validation of a new live cell strain system and have applied it to the
study of plasma membrane stress failure. Guided by images of injured lungs with epithelial gaps and breaks (5, 9, 10), we postulate that plasma membrane stress failure is an important, if not the key, event in the pathogenesis of ventilator-induced lung injury. In contrast to work on cardiac and skeletal muscle, relatively little attention has been given to this problem in the lung field. Future studies should, therefore, focus on two questions of fundamental importance: 1) how does an alveolar epithelial cell deform during breathing, and 2) what determines the stress distribution between CSK and the plasma membrane in alveolar epithelial cells?

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