Mechanisms of surface-tension-induced epithelial cell damage in a model of pulmonary airway reopening

ANASTACIA M. BILEK, KAY C. DEE, AND DONALD P. GAVER III
Department of Biomedical Engineering, Tulane University, New Orleans, Louisiana 70118
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Bilek, Anastacia M., Kay C. Dee, and Donald P. Gaver III. Mechanisms of surface-tension-induced epithelial cell damage in a model of pulmonary airway reopening. J Appl Physiol 94: 770–783, 2003. First published October 25, 2002; 10.1152/japplphysiol.00764.2002.—Airway collapse and reopening due to mechanical ventilation exerts mechanical stress on airway walls and injures surfactant-compromised lungs. The reopening of a collapsed airway was modeled experimentally and computationally by the progression of a semi-infinite bubble in a narrow fluid-occluded channel. The extent of injury caused by bubble progression to pulmonary epithelial cells lining the channel was evaluated. Countervactively, cell damage increased with decreasing opening velocity. The presence of pulmonary surfactant, Infasurf, completely abated the injury. These results support the hypotheses that mechanical stresses associated with airway reopening injure pulmonary epithelial cells and that pulmonary surfactant protects the epithelium from this injury. Computational simulations identified the magnitudes of components of the stress cycle associated with airway reopening (shear stress, pressure, shear stress gradient, or pressure gradient) that may be injurious to the epithelial cells. By comparing these magnitudes to the observed damage, we conclude that the steep pressure gradient near the bubble front was the most likely cause of the observed cellular damage.

Considerable recent interest in ventilator-associated lung injury, including in vitro studies, animal models, and several clinical trials, has predominately focused on the role of high inflation pressures and large tidal volumes. (For recent reviews on this topic, see Refs. 11, 12, 25a). Ventilation at low lung volumes and pressures fosters a different mode of lung injury, in which airway instability leads to repetitive collapse and reopening (11, 12, 25a). During reopening, a finger of air must progress through a collapsed airway, generating stresses on airway walls, potentially damaging airway tissues (2, 15, 33, 54).

Airway collapse and reopening does not occur routinely during normal tidal breathing in the normal lung because surfactant serves to stabilize the airways, preventing collapse. However, lungs in animal models with a normal surfactant system can be exposed to repeated cycles of airway collapse and reopening using negative end-expiratory pressure without injury (46, 47). Even mild surfactant dysfunction leads to severe lung injury, including epithelial destruction, under the same ventilation protocol (46). In situations in which the pulmonary surfactant system of the lung is severely compromised, lung damage occurs with spontaneous breathing or mechanical ventilation (29, 35, 38). Therefore, surfactant may have a “protective” effect when airway collapse and reopening does occur. Although no direct evidence exists, it has been speculated that this damage results from repetitive airway collapse and reopening (36).

The spatial and temporal diversity of acute lung injury and the complex morphology of the lung make evaluation of the specific consequences of particular mechanical stimuli difficult to assess in whole lung models. However, in vitro cell-culture models allow the application of a defined mechanical environment and the identification of a particular cellular response. For example, substrate-stretching devices, used to model airway and alveolar hyperdistension, have demonstrated that mechanical strain can lethally injure pulmonary epithelial cells, slow wound healing, and elicit inflammatory responses (44, 49, 50, 52). Several benchtop and computational models have been developed to represent the mechanical behavior of an individual

Address for reprint requests and other correspondence: D. P. Gaver III, Dept. of Biomedical Engineering, Lindy Boggs Center, Suite 500, Tulane Univ., New Orleans, LA 70118 (E-mail: donald.gaver@tulane.edu).
reopening airway (15, 17, 18, 22, 41, 54). However, an airway reopening model that evaluates the biological response to stresses associated with reopening has not been reported. This work presents two companion studies, an experimental investigation and a computational fluid dynamic simulation, that address the hypothesis that the mechanical stresses associated with airway reopening inflict injury to the pulmonary epithelium.

This investigation had three goals: 1) to create an in vitro model of airway reopening incorporating pulmonary epithelial cells; 2) to quantify pulmonary epithelial cell trauma caused by a single “reopening” event with pulmonary surfactant deficiency; and 3) to evaluate the protective role of pulmonary surfactant during reopening. To accomplish these goals, a parallel-plate flow chamber lined with pulmonary epithelial cells was implemented as an idealized model airway (Fig. 1). The narrow channel of the chamber was filled with an occlusion fluid. Airway reopening was generated by the steady progression of a semi-infinite bubble of air down the length of the channel, which cleared the fluid occlusion, leaving only a thin film on the walls of the channel. Epithelial trauma was assessed by using fluorescent staining methods.

A computational fluid dynamic simulation was developed to analyze and interpret the experimental results. The goals of the fluid dynamic simulations were 1) to describe the mechanical stimulus applied to the epithelial cells in the experimental model; 2) to identify key components of the applied stimulus that may influence the degree of cellular trauma; and 3) to compare the computational and experimental findings to provide insight into mechanism of pulmonary epithelial cell damage in airway reopening. For the fluid dynamic simulation, the bubble and parallel-plate flow chamber were modeled as a semi-infinite bubble progressing within a Hele-Shaw cell. A Hele-Shaw cell is a viscous fluid-filled parallel-plate flow chamber in which the height separating the bottom and top walls is very small compared with the length and width of the chamber. The cross section in the height-length plane can be represented by a long narrow channel. For the experimental conditions, the degree of cellular trauma was compared with the magnitude of several mechanical quantities (the maximal normal and shear stress, the maximal normal and shear gradients, and film thickness) to identify the fluid dynamic features that may influence reopening injury.

Mechanical Stresses During Airway Reopening

Airway closure of the distal airways at low lung volumes has been hypothesized to occur via two possible mechanisms. In one mechanism, “meniscus formation,” a fluid plug spans the lumen of an undeformed airway. This type of occlusion has been modeled (27, 39a), and is demonstrated in vitro by Hubmayr (23). In the other mechanism, “compliant collapse,” the walls of the airway buckle inward, and the lining fluid adheres to the walls. Photomicrographs of occluded airways of isolated canine lungs demonstrate the collapse of distal bronchi (24). Furthermore, mechanical models indicate that airways may buckle to assume a flat “ribbonlike” configuration due to surface tension forces (21).

Figure 2A shows the stress field exerted on the walls as a semi-infinite bubble progresses through a compliantly collapsed airway. In this process, the airway walls are separated in a peeling motion by the bubble progression, as analyzed by Gaver et al. (15) and indirectly observed experimentally (37, 55). Reopening induces large and rapid changes in normal and shear stress along the airway walls. The spatial and temporal gradients of stress exert dynamic, large, and potentially damaging stresses on the airway epithelium that are not typically seen in one-phase steady-flow conditions (15, 26, 54). Not represented in Fig. 2A is tension applied to the cells as result of the walls bending and the hoop stress caused by the distending pressure.

For the present study, a rigid parallel-plate chamber was used as a model of an occluded airway. Using parallel plates simplifies the geometry of the model to an essentially two-dimensional system from which the air-liquid interfacial shape and the fluid dynamical behavior can be computationally evaluated. This representation is suitable for analysis of the clearance of liquid from an uncollapsed airway. In addition, this simplification may represent the tip region of a compliantly collapsed airway, because for very-low-velocity reopening the walls in the region of the bubble tip are nearly parallel (26). Clearly, this model neglects the effects of flexibility, which is discussed in the Limitations section.

The narrow channel model of airway reopening results in a cycle of stresses along the walls similar to the compliant model. Figure 2B shows the hypothetical stresses exerted on the walls as a fluid occlusion is cleared by the progression of a semi-infinite bubble of air, leaving a thin film. The mechanical behavior of this reopening model far ahead and behind the tip of the progressing bubble is simple, and the region near the bubble tip is more complex and time dependent. Far downstream, the cells are exposed to a small shear...
reopening model are the negative pressure that pulls the cells inward as the bubble approaches, the substrate tension associated with wall bending, and the hoop stresses caused by the distending pressure.

The stress cycle associated with airway reopening can be separated into four potentially injurious components: pressure, shear stress, the gradient of shear stress, and the gradient of pressure. An explanation of the potential physical mechanisms by which these components might wound a cell is given in the DISCUSSION. If one of these components is the dominant injury stimulus, then the maximum value of that parameter predicted in the fluid dynamic simulation should correlate with the extent of injury observed in the experimental model. In addition, the stress cycle may be modulated by topological modifications due to the presence of cells because these three-dimensional structures project into the occlusion fluid. Far ahead of the bubble, the height of the cellular protrusion into the fluid is very small compared with the height of the flowing fluid. However, near the perimeter of the bubble cap, the thickness of film deposited along the walls may be exceedingly thin and may be equivalent to the cell protrusion. If the film is sufficiently thin, the stresses experienced by the cells may be significantly greater than those predicted for a flat surface (16). Therefore, the computationally determined film thickness may be used to predict whether stress amplification due to topology might occur.

EXPERIMENTAL METHODS

Cell Culture

A fetal rat pulmonary epithelial cell line (CCL-149, American Type Culture Collection, Manassas, VA) was cultured to confluence on a small (1 cm²), square region of a glass microscope slide (38 × 75 mm). To accomplish this, the section of the microscope slide was isolated by using a 0.4-mm-thick Silastic gasket (Pharmacia, SF Medical, Hudson, MA). Pulmonary epithelial cells were then suspended in a culture medium of Ham’s F12K medium with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA) and plated at 50 × 10⁶ cells/cm² in the isolated region. The slides were incubated in 100-mm petri dishes under standard culture conditions (humidified, 37°C, 5% CO₂-95% air) for 6 h. The gaskets were then removed, and 15 ml of culture medium were added to the petri dishes. The pulmonary epithelial cells were cultured to confluence (5–7 days).

Apparatus

A parallel-plate chamber was constructed as an idealized model of a collapsed segment of an airway in which the walls are held in opposition by a viscous fluid (Fig. 3A). A three-sided separation wall was fixed to the bottom section of the external case, forming a slot. The upper and lower walls of the parallel-plate chamber were formed by two glass microscope slides. The upper wall of the parallel-plate chamber consisted of a glass microscope slide, cultured with lung epithelial cells as described above, seated over the separation wall with a 5-mm-wide, 0.4-mm-thick Silastic gasket to form a tight seal. The cell-free lower wall of the parallel-plate
chamber consisted of a smaller glass microscope slide (25 × 75 mm) placed in the slot created by the separation wall. The result was a 25-mm-wide, 70-mm-long channel separated by a measured gap of 1.7 mm. In general, airway closure is thought to occur at the level of the terminal bronchioles. The diameters of closed airways have been measured in normal lungs to be as small as 0.025 cm and as large as 0.2–0.4 cm (9, 24, 39, 55). The channel height of our experimental model was 0.17 cm, which corresponds to this range. In injured or surfactant-deficient lungs, closure may occur in larger airways.

Figure 3B shows the complete experimental setup. The parallel-plate chamber was submerged in a warm saline bath. A constant-rate syringe pump (kdScientific, New Hope, PA) was used to introduce and propagate the finger of air into the channel via a small circular inlet port in the separation wall. The pressure of the infused air was monitored with a pressure transducer (Omega, Stamford, CT). Fluid driven forward by the bubble progression exited freely into the surrounding saline bath from the open end of the channel. A digital camera mounted above the channel collected sequential overhead images of the progressing bubble used to calculate bubble velocity.

The lower slide of the parallel-plate chamber was fit into place in the separation wall, and the channel was flooded with an occlusion fluid. The upper slide was rinsed gently with PBS, inverted, and rolled into place atop the sealing gasket. The top plate was clamped down, and the assembly was transferred into an empty bath. The inlet port was attached to the syringe pump. The bath was filled with 39°C Ca<sup>2+</sup>,Mg<sup>2+</sup>-free PBS. A small volume of air (2.7 ml) was then infused into the parallel-plate chamber at a rate of either 7 or 70 ml/min. This volume generated a bubble front that completely crossed the region covered with cells and did not reach the end of the channel. The flow rates were selected to achieve bubble velocities of ~0.3 and ~3 cm/s. These reopening speeds correspond to inspiratory flow rates of 60 and 600 ml/s in the terminal bronchioles (30). When the infusion was complete, the apparatus was removed from the saline bath and disassembled. The entire assembly, bubble progression, and disassembly process described took less than 5 min.

As a control condition, slides were rinsed with PBS and placed in a petri dish filled with 39°C PBS for 5 min. Potentially damaging elements of the handling process include stresses associated with assembly and disassembly. Unfortunately, a control in which the apparatus was both assembled and subsequently disassembled introduces an uncontrolled, expanding air-liquid interface when the slides are separated during disassembly, the effects of which cannot be isolated from the purposely applied reopening stimulus. This was not an issue for the experimental slides, because the applied air bolus clears the fluid occlusion, and thus at the time of disassembly the channel is filled with air. Instead, we quantified cell injury (methods described below) due to apparatus assembly only. In this study, the necessary dyes were added to the saline before assembly, the entire chamber was allowed to incubate, and the cell layer was imaged while still in the chamber. No increased injury was observed compared with the nonhandled control (data not shown).

**Generation of Reopening Conditions**

Two occlusion fluids were examined as model airway lining fluids. Phosphate-buffered saline including 0.1 mg/ml CaCl<sub>2</sub> and MgSO<sub>4</sub> (PBS) was used to model a surfactant-deficient (high surface tension) airway lining fluid. A highly surface-active airway lining fluid was approximated by using Infasurf (ONY, Buffalo, NY), a biologically derived pulmonary surfactant. The manufacturer’s solution (35 mg/ml phospholipid concentration) of Infasurf was warmed at 39°C and mixed by three gentle inversions. The experimental solution was prepared by diluting the Infasurf in PBS to a final phospholipid concentration of 1 mg/ml. This concentration is above the critical bulk concentration for Infasurf (18). Both occlusion fluids were warmed at 39°C before use.
Measurement of Bubble Velocity

Sequential overhead images of progressing bubble were captured by use of a digital camera. For each slide, the velocity of the bubble front in the region containing cells was determined from a distance scale and time stamp located in the image. The velocities are reported as the means ± SD for five slides per condition.

Quantification of Cellular Injury

Once removed from the apparatus, the slide was gently rinsed with warm PBS. A 250-µl aliquot of 1.2 µM ethidium homodimer-1 (Eth-1) and 1.2 µM calcein AM (Molecular Probes, Eugene, OR) in PBS was slowly dropped over the cells. The slide was then incubated at 37°C for 2 min. These two dyes used in our study are frequently used to differentiate live from dead cells. Eth-1 is excluded from a cell with an intact cell membrane. If the cell membrane is compromised by injury or death, Eth-1 enters the cell and binds to DNA, producing a red fluorescent nucleus. Calcein AM freely enters the cell and is enzymatically converted into a membrane insoluble product. The accumulation of the fluorescent product produces a diffuse green staining of the cytoplasm in cells with intact cell membranes and functional enzymatic activity.

For each slide, five random fluorescence microscopic images were analyzed. The average number of Eth-1-stained nuclei per field was determined by use of a particle-counting tool from digital image analysis software (Optimus 6.5, Media Cybernetics, Silver Spring, MD). For each slide, the density of injured cells was recorded as the average number of Eth-1-stained nuclei (without regard to the status of calcine AM staining) divided by the area of the field. The data are reported as means ± SD for five slides per condition.

Table 1. Measured and calculated parameter values for the experimental conditions

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Q</th>
<th>γ, dyn/cm</th>
<th>U, cm/s</th>
<th>Ca</th>
<th>(τx)max, dyn/cm²</th>
<th>(dτx/dx)max, dyn/cm²</th>
<th>Δτx, dyn/cm²</th>
<th>ΔP, dyn/cm²</th>
<th>f, μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>Slow</td>
<td>70</td>
<td>0.27 ± 0.01</td>
<td>2.7 ± 10⁻⁵</td>
<td>12.9</td>
<td>0.214</td>
<td>10.7</td>
<td>6.96</td>
<td>348</td>
</tr>
<tr>
<td>Infasurf</td>
<td>Slow</td>
<td>25</td>
<td>0.27 ± 0.02</td>
<td>7.6 ± 10⁻⁵</td>
<td>6.7</td>
<td>0.077</td>
<td>3.8</td>
<td>1.84</td>
<td>92</td>
</tr>
<tr>
<td>PBS</td>
<td>Fast</td>
<td>70</td>
<td>2.70 ± 0.33</td>
<td>2.7 ± 10⁻⁴</td>
<td>29.5</td>
<td>0.216</td>
<td>10.8</td>
<td>3.57</td>
<td>178</td>
</tr>
<tr>
<td>Infasurf</td>
<td>Fast</td>
<td>25</td>
<td>2.42 ± 0.26</td>
<td>6.8 ± 10⁻⁴</td>
<td>14.7</td>
<td>0.078</td>
<td>3.9</td>
<td>0.98</td>
<td>49</td>
</tr>
</tbody>
</table>

Values are means ± SD. PBS, phosphate-buffered saline (with Ca²⁺ and Mg²⁺); Infasurf = 1 mg/ml in PBS, Q, infusion rate (slow = 7 ml/min and fast = 70 ml/min); γ, surface tension; U, velocity; Ca = capillary number; (τx)max, maximum shear stress; (dτx/dx)max, maximum shear stress gradient; Δτx, change in shear stress along the length of a 50-µm cell; ΔP, maximum pressure gradient; ΔP, change in normal stress along the length of a 50-µm cell; f, film thickness. Note that the half-channel width (H) = 0.085 cm and the viscosity (μ) = 0.007 g·s⁻¹·cm⁻¹.

EXPERIMENTAL RESULTS

Apparatus Behavior

Figure 4 shows representative overhead traces of a bubble progressing as a result of a 70 ml/min infusion rate in a saline-occluded channel. Initially, a circular bubble formed at the inlet port. The bubble expanded radially until it reached the width of the channel. A smooth, curved air-liquid interface spanned the width of the channel and moved forward at a constant velocity (Table 1). The velocities for the fast (70 ml/min) and slow (7 ml/min) infusion rates measured 2.70 ± 0.33 and 0.27 ± 0.01 cm/s, respectively, for saline-occluded channels. For Infasurf-occluded channels, the bubble velocities measured 2.42 ± 0.26 and 0.27 ± 0.02 cm/s for the fast and slow infusion rates, respectively.

Typical pressure tracings for the four conditions tested in the present study are shown in Fig. 5. Each pressure trace showed an initial spike in pressure, representing the yield pressure needed to initiate bubble formation at the inlet port, followed by a long nearly-constant-pressure region. The slow decrease in pressure was due to the shortening of the Poiseuille pressure region downstream of the bubble tip. The trace ended with a flat region after the bubble stopped.

Cellular Injury

Figure 6 shows micrographs of representative fields of cells from each of the conditions tested in the present study. An injured cell was defined as a cell with an Eth-1-stained (red) nucleus, regardless of the calcine AM staining (green). Figure 7 shows the average number of injured cells per square centimeter for each condition. The control (slides soaked in PBS) showed few Eth-1-stained nuclei (0.60 ± 0.22 × 10³ injured cells/cm²). For the saline-occluded channels, bubble progression at both velocities produced significant increases in the number of Eth-1-stained nuclei compared with the control. The slower velocity resulted in 39.80 ± 8.76 × 10³ injured cells/cm². The faster bubble velocity produced 12.04 ± 5.91 × 10³ injured cells/cm². The addition of 1 mg/ml Infasurf to the occlusion fluid dramatically reduced the number of Eth-1-stained nuclei. For the Infasurf-occluded channels, the slow ve-
cells/cm², which were similar to the control.

Through a Newtonian
finite bubble, with the semi-infinite Hele-Shaw cell. In this model, the walls were separated as a semi-infinite bubble and parallel-plate flow conditions investigated. As such, the topological influence of the cells that were cultured on the top wall was neglected. Instead, the walls were assumed to be flat, rigid surfaces.

In this analysis, slow viscous flow was assumed so that inertial effects could be ignored. This was justified on the basis of the small Reynolds number corresponding to the flow conditions investigated. As such, the only dimensionless parameter that determined the dynamic response of the system is the dimensionless velocity (the “capillary number,” \( \text{Ca} = \frac{\mu U}{\gamma} \)), representing the relative importance of viscous to surface tension effects on the bubble. In the experiments, \( \text{Ca} \ll 1 \), which implied that surface tension was a dominant characteristic. For a given surface tension, an increase in \( \text{Ca} \) represented an increase in velocity. However, for occlusion fluids of differing surface tensions, variation in \( \text{Ca} \) could reflect the difference in surface tension as well.

The system was simulated by following the analysis of Halpern and Gaver (20). Stokes equations

\[
\nabla P = \mu \nabla^2 \mathbf{u} \quad \text{and} \quad \nabla \cdot \mathbf{u} = 0
\]

were solved by use of the boundary element method. The interfacial stress condition applied at the air-liquid interface was

\[
| \sigma \cdot \hat{n} | = \gamma \kappa \hat{n}
\]

where \( \sigma = -\mathbf{I} + \mu (\nabla \mathbf{u} + (\nabla \mathbf{u})^T) \) is the stress tensor, \( \hat{n} \) is the unit normal, \( \kappa \) is the interfacial curvature, \( P \) is the pressure, \( I \) is the identity matrix, \( u \) is the velocity, and \( T \) represents the matrix transpose. In this formulation, Marangoni stresses that would result from surface tension gradients were ignored. In free-surface problems such as this, the boundary was not prescribed. Instead, the interface evolved until a steady-state shape occurred. For a given \( \text{Ca} \), the system was simulated until a steady-state meniscus had developed and the stress-field and bubble geometry were determined (Fig. 8).

Interfacial Geometry

Figure 8A presents representative interfacial domain shapes for \( \text{Ca} = 5 \times 10^{-2}, 5 \times 10^{-3}, \) and \( 5 \times 10^{-4} \). A thin film region existed far upstream \((x < -2H)\). The thickness of this film becomes extremely small for decreasing \( \text{Ca} \). The curved region between the bubble tip \((x = 0)\) and the thin film was the bubble cap. As \( \text{Ca} \) decreased, the bubble cap became semicircular with a perimeter located at approximately \( x = -H \).

Stress Profiles

Figure 8B presents representative shear stress profiles along the wall for \( \text{Ca} = 5 \times 10^{-2}, 5 \times 10^{-3}, \) and \( 5 \times 10^{-4} \). In the thin film region upstream of the bubble tip \((x < -2H)\), a static film existed and the shear stress was vanishingly small. Downstream \((x > 0)\) the shear stress was small and represented the shear stress due to Poiseuille flow ahead of the bubble. In contrast, the shear stress in the region of the bubble cap was far greater than the shear stress in the upstream and downstream regions. This occurred because fluid must be deposited onto the thin film by squeezing around the bubble cap. The resulting convergence of the streamlines induced large shear stresses in the transition region over the range \(-H < x < 0\).

Figure 8C demonstrates the normal stress exerted on the wall for \( \text{Ca} = 5 \times 10^{-2}, 5 \times 10^{-3}, \) and \( 5 \times 10^{-4} \). In the experimental system, the measured bubble pressure was greater than zero and a weak function of time.
because of shortening of the viscous flow regime (Fig. 5). However, for the fluid dynamic simulations, the reference pressure for the system was the bubble pressure, so $P = 0$ refers to the bubble pressure, which was the maximum pressure in the system. Far upstream of the bubble tip ($x < -2H$), the pressure was approximately uniform. In contrast, downstream of the bubble tip ($x > 0$), the pressure decreased linearly with distance. For small $Ca$, this pressure gradient was very small and represented the pressure gradient necessary to drive Poiseuille flow ahead of the bubble. Within the transition region between the thin stagnant film and the semispherical cap, a large change in pressure occurred over $-2H < x < 0$, with the largest pressure gradient occurring at $x \approx -H$. As $Ca$ was reduced, the pressure profile approached that for a static bubble ($Ca = 0$), where the spherical cap would meet the wall at a contact point at $x = -H$, resulting in a pressure discontinuity of magnitude $\Delta P = \gamma/H$.

**Predictions of Maximal Stress Magnitudes During Reopening**

The data from the fluid dynamic simulations confirm that bubble progression in a narrow channel imparts a complex cycle of stresses on the walls of the channel. A number of features of the stress cycle may be physically damaging to the cells and, therefore, relevant to the experimental results. From the computational representation described above, several key features were analyzed: the maximal shear stress $[(\tau_\alpha)_{\text{max}}]$, the maximal shear stress gradient $[(d\tau_\alpha/dx)_{\text{max}}]$, the maximal pressure $(P_{\text{max}})$, the maximal pressure gradient $[(dP/dx)_{\text{max}}]$, and the film thickness $(f)$, and calculated values for these parameters were compared with the observed degree of injury for the experimental conditions.

**Regression Equations**

The $Ca$ for the experimental flow conditions, presented in Table 1 existed over the range $3 \times 10^{-6} \leq Ca \leq 1 \times 10^{-3}$. In some cases, this range extended beyond that feasible for our simulations because the film thickness becomes exceedingly thin (1% of the...
channel width), which results in numerical instabilities of the boundary element method. Nevertheless, we were able to increase the node distribution to reach velocities at the upper end of the experimental range. To predict the stresses and stress gradients relevant to the experiments, regression relationships describing the behavior as a function of Ca were fit for very small Ca. The regression formulae were motivated by asymptotic analysis by Bretherton (8) and Park and Homsy (40).

Shear stress. Figure 9A represents \( (\tau_s)_{\text{max}} \) vs. Ca over \( 5 \times 10^{-4} \leq \text{Ca} \leq 0.75 \). When Ca \( \ll 1 \), asymptotic analysis indicated that \((\tau_s)_{\text{max}} = A \text{Ca}^B \) with \( B = 1/3 \), where A is an undetermined coefficient. Regressing the simulation data to this form over the range \( 5 \times 10^{-4} \leq \text{Ca} \leq 4 \times 10^{-3} \) revealed

\[
(\tau_s)_{\text{max}} = 0.69\text{Ca}^{0.36}
\]

with \( R^2 = 0.9997 \). This regression demonstrated an excellent fit to the simulation data and was consistent with asymptotic analysis.

Shear stress gradient. Figure 9B presents the relationship between \((d\tau_s/dx)_{\text{max}} \) and Ca. Asymptotic analysis suggested that \((d\tau_s/dx)_{\text{max}} \propto \text{Ca}^{1/3} \) for Ca \( \ll 1 \). Regressing the simulation data following this relationship revealed

\[
(d\tau_s/dx)_{\text{max}} = 0.22 + 1.2\text{Ca}^{0.75}
\]

with \( R^2 = 0.999 \) over \( 5 \times 10^{-4} \leq \text{Ca} \leq 0.75 \).

Pressure. The maximal pressure in the simulation was the upstream gas pressure in the bubble, and by definition \( P_{\text{max}}/(\gamma/\text{H}) = 0 \) for all experimental conditions. Therefore, a regression analysis of pressure was not necessary. As will be discussed below, in this system the upstream pressure increases with velocity, which will provide insight of the importance of the magnitude of pressure to cell damage.

Pressure gradient. Figure 9C presents \((dP/dx)_{\text{max}} \) as a function of Ca, which clearly indicates the trend of increasing \((dP/dx)_{\text{max}} \) with decreasing Ca. Asymptotic analysis of this system at small Ca suggested that \((dP/dx)_{\text{max}} \propto \text{Ca}^{1/3} \), with \( B = -1/3 \). Regression analysis of the simulation data using this relationship over the range \( 5 \times 10^{-4} \leq \text{Ca} \leq 2 \times 10^{-3} \) revealed

\[
(dP/dx)_{\text{max}} = 0.34\text{Ca}^{-0.29}
\]

with \( R^2 = 0.9995 \). This form clearly fit the simulation data and was consistent with asymptotic analysis.

Film thickness. Figure 9D presents a regression of the predictions of \( f \) following the form \( f = A \text{Ca}^{B} \). Asymptotic analysis by Bretherton (8) gives \( B = 2/3 \) for Ca \( \ll 1 \). Our regression over \( 5 \times 10^{-4} \leq \text{Ca} \leq 4 \times 10^{-3} \) provided

\[
\frac{f}{H} = 1.03\text{Ca}^{0.63}
\]

with \( R^2 = 0.9999 \). This form fit the simulation data and was consistent with asymptotic analysis.

Predicted Values of Maximal Stress Magnitudes and Film Thickness

By using the regression relationships (Eqs. 3–6), the maximum stress magnitudes and the residual film
thickness were calculated for each of the four experimental conditions (Table 1). The surface tension of the surfactant-free occlusion fluid (PBS) was measured and found to be similar to that of water (70 dyn/cm). The equilibrium surface tension of the Infasurf solution was measured to be 25 dyn/cm, which is consistent with values measured by other researchers for the equilibrium surface tension of Infasurf (18). For both occlusion fluids, the viscosity was measured and found to be similar to that of water (0.007 g·s⁻¹·cm⁻¹). Finally, the half-height of the channel, H, equaled that of the experimental parallel-plate flow chamber (0.085 cm). To calculate the fore-aft tangential- and normal-stress differences experienced by a cell on the channel wall, we approximated a cell length of 50 μm, as estimated from direct measurements, which is appropriate for the cell line used in the experimental studies. Note that airway epithelial cells may be much smaller in vivo. This estimate will thus be accurate when the airway dimension is much larger than the cell length.

DISCUSSION

The experimental results (Figs. 6 and 7) demonstrate that semi-infinite bubble progression in a saline-occluded channel, which was used to model a surfactant-deficient airway, inflicted extensive injury to pulmonary epithelial cells. The addition of 1 mg/ml of Infasurf (highly surface-active, biologically derived pulmonary surfactant used in replacement therapy for infants with respiratory distress syndrome) to the saline occlusion fluid abated the cellular trauma caused by bubble progression, which strongly supports the hypothesis that pulmonary surfactant in the normal lung protects the epithelium from injury due to airway reopening.

Observed Injury May Indicate Plasma Membrane Disruptions

Mechanical stresses initiate and regulate a host of cellular responses in lung epithelial cells (31). Although some of these responses may be deleterious, this study focused on immediately observable cellular injury, which is likely to be due to direct trauma inflicted by the mechanical forces associated with bubble progression. Substrate stretch-induced injuries of pulmonary epithelial cells have been suggested to occur as a result of stretching the plasma membrane beyond its capacity to compensate, causing small breaks (11, 51). If the disruptions are small, they will reseal and the cell may recover. Although the substrate in our model does not impart a stretch on the whole cell, mechanical stresses from the fluid flow may stretch the plasma membrane either directly or as a consequence of cellular deformation. Although these

Fig. 9. Simulation data (●) and regression equations (thin line) for the dimensionless maximal shear stress (τ²max), shear stress gradient ([τ²/τ⁺]max), pressure gradient ([dP/dx]max), and film thickness (f) as a function of Ca. The Ca relevant to the experimental conditions are located in the shaded region. U, velocity; μ, viscosity.
stretches are likely to be small, they would be rapid and perhaps beyond a cell’s ability to compensate. As such, the loss of membrane integrity, demonstrated by the entrance of Eth-1 into the cells, could be the result of the passive interaction of mechanical forces with the three-dimensional structure of the cell. Specifically, the experimental findings suggest that the cycle of mechanical stresses associated with airway reopening cause small plasma membrane disruptions.

In the experimental model, most of the injured cells (Eth-1 stained) also exhibited some calcein AM staining. In general, the fluorescent stains are used to differentiate live from dead cells, because in a given population individual cells are usually effectively stained by only one of the dyes (14, 49). An intact cell membrane is necessary for effective calcein AM staining, whereas Eth-1 is ordinarily excluded from a cell with an intact membrane. Therefore, the double staining reflects a state of cellular trauma during the staining period (~30–150 s postbubble progression) in which the plasma membrane is both compromised (allowing Eth-1 to enter) and functional (concentrating calcein AM). Although it is conceivable that both cell membrane integrity states existed simultaneously, the more likely explanation is that early in the staining period the cell membrane was compromised and then resealed. This finding is consistent with other studies of plasma membrane disruptions, which have been shown to reseal in ~5–120 s depending on the size of the rupture (32, 45). As such, the double staining finding suggests that the injury inflicted by a single reopening event may not be lethal.

Comparison of Experimental and Theoretical Results

The fluid dynamic simulations revealed that hydrodynamic environment of the cells in this model is complex and suggested that different components of the stress cycle associated with airway reopening may be injurious. More specifically, the injury marker used in the experimental study suggests that one or more of the components of the stress cycle may cause plasma membrane disruptions. The stress cycle can be separated into four potentially injurious components: shear stress, the gradient of shear stress, pressure, and the gradient of pressure. An explanation of the physical manner in which each stress cycle component may compromise the cell membrane is described below. If one of the components is the dominating mode of cellular injury during reopening, then the maximum value of the parameter should correlate to the extent of injury to the cell population observed in the experimental model. The parameter should correlate to two observations. First, the degree of trauma for the slow velocity should be predicted to be greater than for the high-velocity bubble progression in saline-occluded channels, which is an interesting and counterintuitive result. Second, for both the slow and fast velocities, the degree of injury for the Infasurf-occluded channels must be predicted to be significantly less than that predicted for saline-occluded channels. A comparison of the experimental and theoretical observations is given below for each stress cycle component. As will be demonstrated, the only stress component that is consistent with these observations is the maximal pressure gradient, \( \frac{dP}{dx} \)max.

Shear stress. Shear stress exerts tractional stress over the surface of a cell that might induce deformations of the cell, causing plasma membrane disruptions. Additionally, shear stresses can be translated from the surrounding fluid to the nontethered components of the cell membrane. The cell membrane may be “rarefied” in certain regions because of shifting of the nontethered components and thus become more susceptible to tearing (51). The maximal shear stress predictions (Table 1) suggest that if the shear stress magnitude induces the damage, the greatest degree of injury should occur with fast-velocity bubble progression in saline-occluded channels. Additionally, fast-velocity bubble progression in Infasurf-occluded channels and slow-velocity bubble progression in saline-occluded channels should induce nearly identical damage because \( \tau_{\text{max}} \sim 15 \text{ dyn/cm}^2 \) in each case. These predictions are inconsistent with the experimental observations, and thus the shear stress magnitude is discounted as the predominant mechanism of injury investigated in this study.

Shear stress gradient. Shear stress gradients cause force imbalances within the plane of the cell membrane, directly increasing the tension of the cell membrane. Cell membranes can carry only a very small tension (51) and may rupture under gradients of shear stress. The predicted maximal shear stress gradients and estimated change in shear stress over the length of a cell were larger for the saline-occluded channels than for the Infasurf-occluded channels, which could explain the differences in cellular damage observed for the two occlusion fluids. However, for each occlusion fluid the shear stress gradient sensitivity to velocity was insignificant. Therefore, the shear stress gradient cannot explain the observed reduction in damage with increasing velocity in the saline-occluded channels. Therefore, the shear-stress gradient is not the dominating mode of injury in our experimental model of airway reopening.

Pressure. Maximum pressure, as occurs in the region of the patent airway, exerts an evenly distributed normal stress on the cells. Although studies have shown that a uniform pressure stimulus alters the behavior of some cells (1, 42), it is unlikely that this stimulus can directly compromise the cell membrane because the transmural pressure will equilibrate at the transmission speed of an acoustic wave. Furthermore, as seen in Fig. 5, the maximum reopening pressure increases with velocity, which is in direct contrast to the observed cellular damage. Therefore, the maximal pressure is unlikely to be the dominant physical mechanism of cellular injury in the experimental model of airway reopening.

Pressure gradient. Pressure gradients create normal stress imbalances on the cell membrane over the length of the cell. For a low profile, predominately flat region of a cell, the nonuniformly distributed load may
depress and stretch regions of the membrane. In addition, we speculate that the normal-stress difference could induce transient internal flows within the cell that could exert hydrodynamic stresses on the intracellular surface of the cell membrane, which might injure the membrane by the same mechanisms as extracellular stresses. High-profile cells or regions of a cell, for example due to the protrusion of the cell nucleus, will result in net normal forces on either side of the region that act in opposition; thus a pressure gradient will pinch that region. The pinching could tear the membrane at the base of the protrusion or force fluid upward, rupturing the top surface of the cell. The results of our simulation show that slow-velocity bubble progression in saline-occluded channels generated large pressure gradients (and differences) along the cell body. These gradients are much larger than those necessary to drive Poiseuille flow at the same rate (see downstream region of Fig. 8C). Our simulations show that increasing the velocity reduces the pressure gradient (and difference), which is consistent with the reduction of cell damage that was observed in the saline-occluded channels. Furthermore, the pressure gradient is greatly reduced (by nearly a factor of four) by the introduction of surfactant to the occlusion fluid. These predictions are consistent with the experimental observations and thus strongly support a pressure-gradient-induced damage hypothesis. It should be noted that, unlike the cells used in the present study, most of the airway epithelium in vivo is pseudostratified or cuboidal, leading to a relatively smooth surface. However, microstructural variations within the network of branching airways can be influenced by hydrodynamic stresses in the same manner as that discussed above.

Influence of topology. Fluid flow near the perimeter of the bubble cap is restricted to the narrow space between the air-liquid interface and the wall of the channel. For a flat, rigid wall, the close proximity of the moving air-liquid interface dramatically increases the flow resistance and is responsible for the peak in stresses demonstrated in the simulation data (Fig. 8B). In the experimental model, one wall of the channel is covered with pulmonary epithelial cells, which protrude into the moving fluid. If the film thickness near the perimeter of the bubble cap approaches the height of these protrusions, the flow resistance and stresses may be further amplified. Gaver and Kute (16) examined the effect of a semicircular protrusion in a narrow channel on the stress profiles along the walls created by one-phase steady flow. They demonstrated that bulges with radii of >25% of the channel width substantially amplified the maximal shear stress. Similarly, the presence of a bulge in the wall amplified the gradients of shear stress and pressure (16). In our reopening model, if the height of a cell protrusion due to the nucleus is ~5 μm, the stress field caused by bubble progression may be amplified when the film thickness is <20 μm. The estimated film thicknesses for the four experimental conditions in the present study are within this range. The film thickness for increasing velocity, thereby decreasing the flow resistance and stress amplification, which may in part explain the decrease in cellular trauma observed for the fast velocity in the saline-occluded channels compared with the slow velocity. However, the film thicknesses for the Infasurf-occluded channels, although larger for each velocity, are still within the range that could cause stress amplification. Our simulation assumed a constant surface tension along the air-liquid interface. However, surfactants in similar flow conditions are not evenly distributed on the interface, creating Maragoni stresses that drive fluid into the thin film (18, 54). As such, the film thicknesses for the Infasurf-occluded channels may be underestimated by our flow model. Therefore, the interaction of the air-liquid interface with the irregular surface of the cell population may still be an important factor in airway reopening.

Limitations

Although the pressure gradient best predicts the degree of injury to the cell population, the individual components within the stress cycle do not act independently. For example, the maximal pressure gradient occurs in conjunction with a large shear stress (Fig. 8). As the body of the cell is pinched by the pressure gradient, the high shear stress may additionally rarefy the top of the cell membrane and make it more susceptible to rupture.

The time dependence of the applied stimulus could also be important to the observed injury. Velocity determines both the temporal gradients of stress and the duration of exposure to an individual cell. For a given spatial gradient of stress (dσ/dx), the temporal gradient dσ/dt = Uρ dσ/dx = −Ca × dσ/dx. Therefore, from the relationships provided by Eqs. 4 and 5, the temporal gradients of both shear stress and pressure increase with bubble velocity, which does not correlate with the observed damage. In addition, as the velocity increases, the cell has less time to actively compensate to the new stress load on the cell membrane. Therefore, although one might hypothesize that damage should increase with increasing temporal gradients of stress, the cellular response is inconsistent with this hypothesis. It remains possible that slow reopening causes increased damage by exposing cells to mechanical stress for a longer duration of time. The present study cannot establish this relationship, because this would require the simultaneous modification of fluid properties and airway geometry to independently vary the stress magnitude and exposure time.

Although only the magnitude of the spatial pressure gradient could be directly related to the cell damage, it is conceivable that other stress components could be responsible for damage if the duration of stress exposure is also a factor in the injury mechanism. For example, our slow bubble progression experiments exposed the cells to the stress cycle components 10 times longer than the fast bubble progression. Excessive duration of high stress could exhaust a cell’s capacity to adapt to the mechanical stress. This might explain the

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reduction of damage that occurred with the Infasurf-occluded channels compared with the saline-occluded channels. However, insignificant damage occurred during trials using slow-velocity reopening in Infasurf-occluded channels, which suggests that duration alone is not the critical element necessary to inflict stress-induced damage. It is possible that for different durations of exposure, different critical levels of stress are needed to induce damage. For example, in the saline-occluded channels, the critical shear stress magnitude might be above \( \sim 8 \, \text{dyn/cm}^2 \) for the slow-velocity (long-duration) experiments and above \( \sim 17 \, \text{dyn/cm}^2 \) for the fast-velocity (short-duration) experiments. Alternatively, the slower velocity exposes the cell to the stimulus for a longer duration, which may create larger membrane disruptions that could take longer to reseal. Within our experimental methods, a finite time lag was needed to disassemble the apparatus before the exposure to the dyes. Therefore, it is conceivable that the observed difference in the number of Eth-1 stained cells could reflect a difference in the size of the disruptions rather than the number of ruptured cells. Although this represents a potential limitation of the experimental methods and analysis, the insignificant damage inflicted during slow-velocity trials in Infasurf-occluded channels again suggests that duration alone is not the critical element.

There are several deviations between the experimental studies and in vivo airway reopening that might influence the validity of our results. First, the morphology of the pulmonary epithelium in vivo differs from that used in our experiments. In general, airway epithelium in Airways with a diameter similar to our channel height (0.17 cm) is pseudostratified or cuboidal with a relatively smooth surface. The pulmonary epithelial cells used in our experiments have a larger (40- to 60-\( \mu \)m diameter), flattened morphology with nuclear bulges. The lack of large topographical variations may reduce the in vivo airway epithelium’s susceptibility to injury. However, other topographical variations in vivo due to geometrical characteristics of Airways are likely to exacerbate cell damage beyond what would exist for flattened cells in our idealized flow chamber.

Another deviation from in vivo conditions occurs because of flexibility of the substrate. In our experimental model, the cells are cultured on a rigid glass slide. In the lung, the pulmonary epithelium grows on a flexible basement membrane surrounded by compressible tissue, both of which deform during reopening. The rigidity of the substrate alters the stress stimulus applied to the cells. In particular, Fig. 2A shows that flexibility induces a significant inward normal stress downstream of the bubble tip (15). This additional stimulus is removed in our rigid-walled model, Fig. 2B. Hubmayr (23) suggests that this flexibility will focus stresses if compliant collapse occurs. Thus flexibility might induce greater damage in vivo compared with that observed in our experiments. Alternatively, with a rigid substrate all deformations caused by the applied stimulus are constrained to the cells. Therefore, it is possible that wall flexibility in vivo could protect cells by allowing the stresses to be distributed more fully to surrounding structures. Clearly, the impact of flexibility is an aspect that should be studied further but is beyond the scope of the present investigation.

Our experiments have been designed to model airway reopening. However, Hubmayr (23) contends that in the acute respiratory distress syndrome lung, airway closure may be due to liquid bridge formation and the trapping of gas bubbles in noncollapsed Airways. In that case, reopening may occur either by the progression of the meniscus or by meniscus rupture. The findings of our study are relevant only to the bubble progression mechanism.

Finally, the high reopening pressures seen in vivo (37) could impose a hoop stress \( (\tau_{\text{hoop}}) \) on the patent segments of the airway if transmural pressures are large. We estimate that if the surrounding Airways are completely collapsed, \( \tau_{\text{hoop}} \) could have a magnitude of as large as \( 10^5 \, \text{dyn/cm}^2 \), which could induce cell injury. However, in general we expect transmural pressures to be far smaller because of potency of surrounding Airways and parenchymal tethering. In addition, epithelial cells are backed by a strong basement membrane that will carry the mechanical load of the hoop tension, as hypothesized for pulmonary capillaries by West and Mathieu-Costello (53). Finally, at the cellular level, hydrodynamic and hoop stresses will influence cells differently. The hydrodynamic stresses are applied to the apical surface of the cell. In contrast, the hoop stress results from a circumferential tension distributed across the thickness of the cell. Therefore, hoop stress generated during reopening in vivo constitutes an additional mechanism for epithelial injury that is beyond the scope of the present study.

Potential Consequences

The experimental methods of this study quantified the degree of injury to the population of pulmonary epithelial cells immediately after bubble progression. Although not specifically assessed, several potential consequences of the observed injury, important to the known characteristics of acute lung injury, can be inferred. Because cells have active mechanisms to repair structural damage, the injured cells may recover. For example, small plasma membrane disruptions are repaired via a vesicle fusing mechanism similar to exocytosis (4, 45). The double staining (Eth-1 and calcein AM) of the injured cells in the experimental model suggests that the pulmonary epithelial cells in our airway reopening model are alive and recovering. However, the cells may ultimately die as result of the mechanical injury. Additionally, this study examined the injury caused by a single reopening event. Repeated cycles of bubble progression and regression completely denude the surface of the narrow channel (unpublished results). As such, airway reopening may contribute to the focal airway and alveolar epithelial destruction observed in acute lung injury (10, 25, 28, 34, 46).
A critically important potential consequence of the observed epithelial injury is the activation of inflammatory mechanisms in the recovering injured or surrounding uninjured cells. The early stage of acute lung injury is characterized by extensive and progressively worsening inflammation of the airways and alveolar structures (10, 25, 28, 34, 46). Elevated concentrations of inflammatory mediators are often found in the bronchial alveolar lavage fluid and plasma during acute lung injury (10–12, 48). Pulmonary epithelial cells release inflammatory mediators in response to substrate stretch (31, 52), which may be one source of the cytokine elevations observed during mechanical ventilation with large tidal volumes. Similarly, increased concentrations of inflammatory mediators were found in the alveolar lavage fluid of animals mechanically ventilated with moderate tidal volumes and zero end-expiratory pressures, which is conducive to airway collapse and reopening (48).

Grembowicz et al. (19) demonstrated that plasma membrane disruptions increase expression of c-fos and the translocation of nuclear factor-kB. These transcription factors are important regulators of inflammation (5, 11). If plasma membrane disruptions occur as a result of the mechanical stresses associated with airway reopening, then the activation of inflammatory mechanisms may occur in the injured cells. Additionally, injured cells may communicate with surrounding cells via intra- and extracellular mechanisms (43). For example, wounding a cell monolayer by scratching with a stylus induced calcium waves that propagated through the surrounding uninjured cells (43). Many biochemical activities, including the regulation of inflammatory mechanisms, are calcium regulated and may be influenced by intercellular communication mechanisms (11, 31). Accordingly, the injured epithelial cells in our airway reopening model may induce responses in the surrounding uninjured cells.

In conclusion, the progression of a semi-infinite bubble in a narrow channel lined with pulmonary epithelial cells inflicts significant injury to epithelial cell population. The presence of pulmonary surfactant effectively abates this injury. The complex hydrodynamics of the bubble progression in this model influence the degree of injury. The computational fluid dynamic simulations demonstrate that the most mechanically damaging element of the stress cycle associated with bubble progression was the steep pressure gradient near the perimeter of the bubble cap. This study addressed immediate cellular trauma caused by the bubble progression. It does not address the survivability of this injury nor does it specifically address other potential responses of the cells, such as the upregulation of inflammatory mediators. Although the present study was posed to address a hypothesis specific to airway reopening, the findings may be relevant to other problems, including arteriolar gas embolism (7). Furthermore, the results reflect the value of the coupled experimental and computational models in future investigations of airway reopening.

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