Viscoelasticity of human alveolar epithelial cells subjected to stretch

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Submitted 25 March 2004; accepted in final form 7 July 2004

Trepát, Xavier, Mireia Grubulosa, Ferranda Puig, Geoffrey N. Maksym, Daniel Navajas, and Ramon Farré. Viscoelasticity of human alveolar epithelial cells subjected to stretch. Am J Physiol Lung Cell Mol Physiol 287: L1025–L1034, 2004. First published July 9, 2004; doi:10.1152/ajplung.00077.2004.—Alveolar epithelial cells undergo stretching during breathing and mechanical ventilation. Stretch can modify cell viscoelastic properties, which may compromise the balance of forces in the alveolar epithelium. We studied the viscoelasticity of alveolar epithelial cells (A549) subjected to equibiaxial distention with a novel experimental approach. Cells were cultured on flexible substrates and subjected to stepwise deformations of up to 17% with a device built on an inverted microscope. Simultaneously, cell storage (G”) and loss (G’) moduli were measured (0.1–100 Hz) with optical magnetic twisting cytometry. G’ increased with strain up to 64 and 30%, respectively, resulting in a decrease in G” following a power law with exponent α = 0.197. G” increased proportionally to G’ but exhibited a more marked frequency dependence at high frequencies. Stretching (14%) caused a fall in α (13%). At high stretching amplitudes, actual cell strain (14.4%) was lower than the applied substrate strain (17.3%), which could indicate a partial cell detachment. These data suggest that cytoskeletal prestress modulates the elastic and frictional properties of alveolar epithelial cells in a coupled manner, according to soft glassy rheology. Stretch-induced cell stiffening could compromise the balance of forces at the cell-cell and cell-matrix adhesions.

ADHERENT LIVING CELLS EXPERIENCE a variety of mechanical stresses that regulate fundamental cellular functions such as growth, spreading, migration, mechanotransduction, differentiation, apoptosis, and protein synthesis (12, 27, 29). Mechanical stresses and the resulting deformations are particularly relevant in alveolar epithelial cells, which undergo considerable stretch during breathing. Although the levels of strain experienced by alveolar epithelial cells in vivo have not yet been well characterized, a number of studies have reported that the alveolar basement membrane surface area increases by 15–35% close to total lung capacity (TLC) (4, 34, 51). This stretch may be dramatically raised in injured lungs receiving mechanical ventilation as recently suggested by in vivo video-microscopy in an animal model (46). Maintaining the integrity of the alveolar epithelium during stretching requires cytoskeletal tension to be balanced by the tethering forces at cell-cell and cell-extracellular matrix adhesions. Therefore, knowledge of the mechanical response of alveolar epithelial cells to stretch is important for better understanding the structure and function of the alveolar barrier under pathophysiological conditions.

Few data are available on the effect of stretching on the mechanical properties of adherent cells. Pourati and coworkers (37) reported that subjecting bovine endothelial cells to small amplitude stretching (~5%) induced an increase in apparent cell stiffness. This stretch-induced stiffening was attributed to an increase in cytoskeletal tension (prestress). Such behavior was interpreted on the basis of models of stress-supported structures. In these models, cell prestress is carried by actin and intermediate filaments and balanced internally by microtubules and externally by cell-cell and cell-matrix adhesions. Pourati and coworkers (37) interpreted the observed stretch-induced stiffening on the assumption of a purely elastic cellular behavior.

There is solid evidence, however, that cells are viscoelastic, i.e., they store and dissipate energy (7, 33, 48, 60). One common approach to characterize cell viscoelasticity is to compute its complex elastic modulus (G”) from oscillatory measurements, defined as the complex ratio between applied mechanical stress and resulting strain. G” has two components, the storage modulus (G’) and the loss modulus (G”), which account for the solid-like elastic behavior of the cell and for its liquid-like dissipative behavior, respectively. It has been reported that G” exhibit smooth positive frequency dependence over a wide bandwidth without displaying plateaus, shoulders, or inflections, thereby showing no characteristic resonant frequencies or relaxation times (2, 19, 38, 61). Recently, Fabry and coworkers (19, 20) interpreted the cell viscoelastic behavior in terms of the rheology of soft glassy materials (SGMs). This approach assumes that elastic and frictional stresses in the cell are coupled within the cytoskeleton and that they are not dominated by any particular resonant frequency. The cell viscoelastic response to different pharmacological interventions has been studied for a variety of cell types (19, 33, 60, 61). However, no study has examined the changes in cell viscoelasticity due to mechanical stretch and whether the response can be interpreted in terms of the aforementioned models of cell mechanics.

The aim of this work was to study the viscoelastic properties of human alveolar epithelial cells (A549) subjected to equibiaxial distention. We developed a novel experimental approach to stretch cells cultured on elastic substrates and to simultaneously measure cell viscoelasticity. Cell stretching was produced by distending the substrate with a vacuum-driven device...
mounted on an inverted microscope. The cell G* was measured with an optical magnetic twisting cytometer coupled to the stretching device. This technique is based on binding ferrimagnetic microbeads to the cell surface and on subsequently twisting them in a sinusoidal magnetic field (22). We also assessed the transmission of strain from the elastic substrate to the underlying cells. Measurements were carried out over a broad twisting frequency range (0.1–100 Hz) for stepwise deformations up to 17%.

MATERIALS AND METHODS

Cell culture and sample preparation. Human alveolar epithelial cells A549, culture line CCL-185 (ATCC, Manassas, VA), were used. Cells were cultured in HEPES-buffered RPMI 1640 (GIBCO, Gaithersburg, MD) with 10% inactivated fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel), 1 mM l-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (GIBCO), and 2 μg/ml amphotericin B (Bristol-Myers Squibb, New Brunswick, NJ). For 3 experiments, cells were harvested with a brief exposure to trypsin. For coating ferrimagnetic beads (Fe3 O 4, 400 nM; Flexcell International), Measurements were performed at confluence 48 h after plating. Coated ferrimagnetic beads with a short and strong magnetic pulse. The pair coaxial to the optical magnetic twisting cytometer coupled to the stretching device. This technique is based on binding ferrimagnetic microbeads to the cell surface and on measuring the resulting bead displacement with videomicroscopy. We used two orthogonal pairs of coaxial coils coupled to the stretching device to magnetize and subsequently stretch the beads (Fig. 1). Permanent magnetization of the beads in the horizontal direction was achieved with a brief (20 ms) and large (120 ms) twist the beads (Fig. 1). Permanent magnetization of the beads in the horizontal direction was achieved with a brief (20 ms) and large (120 ms) pulse of magnetic field produced by discharging a capacitor (1 mF, 430 V) into the magnetizing pair (radius 36 mm and separation 72 mm). The twisting field was produced by a second pair of coils (radius 23 mm and separation 34 mm) coaxial with the optical axis of the microscope. The twisting coils were fed with a sinusoidal current of up to 3-A amplitude. The magnetizing and twisting fields were homogeneous (<5% variation) within the sample.

Imaging was performed with the CCD camera controlled with an external trigger. The apparent pixel size after magnification (×10) was 820 nm with a resulting field of view of 640 × 480 μm (Fig. 2). The electronic shutter speed of the camera was set to 0.1 ms. Heterodyne acquisition was used at twisting frequencies >10 Hz (19). The analog video signal was digitized and transferred to the personal computer memory by an eight-bit resolution frame grabber (PC Eye4; Eltec, Mainz, Germany). Both the current fed to the coils and the camera trigger were controlled with an analog-digital/digital-analog peripheral component interconnect board (PCI-MIO-16XE-10; National Instruments, Austin, TX) driven by LabVIEW software (National Instruments).

Measurements. The first set of viscoelasticity measurements was aimed at determining the cell rheological response to substrate deformations of various amplitudes. The beams were twisted for 10 s by applying an oscillatory magnetic field of 2-mT amplitude at 1 Hz under unstretched conditions (baseline). A stretch of the substrate was produced, and the beams were twisted as previously 1 min after stretching. Stretch was held, and the oscillation was repeated 5 min later. Finally, the substrate was relaxed to its initial unstretched conformation, and the beams were twisted 1 min after relaxation. The beams were magnetized before the baseline measurement and after stretching or releasing the substrate to ensure that the magnetic moment was kept aligned throughout the experiments. The image was focused manually before twisting. This series of measurements was
done in eight wells for 6.5% strain and in another eight wells for 17% strain.

We assessed the role of the actin cytoskeleton in the cell response to stretch in latrunculin A-pretreated cells (n = 7 wells). Latrunculin A (Calbiochem, La Jolla, CA) was added to the well (final concentration of 1 μM) 20 min after bead addition. After a further 30 min, the beads were oscillated under baseline conditions and after application of a stepwise deformation [14% substrate strain (SS)]. The beads were twisted with a weaker field (0.2 mT) to keep the bead displacement amplitude within the same range (~100 nm).

In a third series of experiments, we studied whether the measurement of the stretch-induced response was dependent on the specificity of the bead coating. Beads coated with acLDL were added to the wells (n = 7 wells). Unlike RGD-coated beads, which bind tightly to the cytoskeleton, acLDL-coated beads bind to low-density lipoprotein receptors of the cell membrane, which do not form focal adhesions (8, 56). Therefore, measurements with acLDL-coated beads allowed us to ascertain whether the observed response was determined by the mechanics of the cell surface or by that of the underlying cytoskeleton. After 20 min, the beads were twisted (0.2 mT) under baseline conditions and after applying a stepwise deformation (14% SS).

Finally, the frequency dependence of cell viscoelasticity was measured for an SS of 14%. The beads were twisted (6 mT) for 10 cycles (10 frames/cycle) at 0.1, 1, 11, and 101 Hz in random order. Measurements were carried out under baseline conditions and 1 min after applying the stretching step.

Data processing. Image analysis was performed with a multiparticle tracking application developed in Visual C++ 5.0 and described elsewhere in detail (49). The first frame of each oscillatory measurement was processed to identify the beads according to a set of user-defined geometrical parameters (bead size, shape, and contrast, minimum distance between beads) and thresholds. The bead position was measured with nanometer resolution and tracked through the subsequent frames using a centroid algorithm. After computing the position of each particle through the sequence, we applied a moving average digital filter with a time window of one oscillation period to the tracking signal.

To evaluate the transmission of strain from substrate to cells, we compared the SS obtained from the device calibration described above with the actual cellular strain quantified as follows. Two cellular strain indexes were computed to account for intercellular and intracellular strain. Intercellular strain was calculated as the fractional length change of the line elements between beads that were at least 100 pixels (82 μm) apart from each other. This strain index was indicative of the actual bulk deformation of the cell monolayer and was termed cell layer strain (LS). Intracellular strain was measured as the fractional length change of the line elements between beads that were bound to the same cell. This index was averaged over at least 15 cells in each well and was taken as the actual cell strain (CS).

We computed the specific torque (T) applied to a bead as:

$$T = mB/V$$

where V is the bead volume, m is the bead magnetic moment, and B is the applied magnetic field [the variation of the angle between the magnetic moment and the applied twisting field was neglected (22, 33)]. An effective G*(ω) of the cells was computed from the Fourier transforms of the applied torque [T*(ω)] and of the resulting bead displacement [d*(ω)]

$$G*(ω) = G'(ω) + jG''(ω) = \frac{T*(ω)}{d*(ω)}$$

where G'(ω) we define as a storage modulus, G''(ω) we define as a loss modulus, ω is the radian frequency, and j is the imaginary unit defined as j^2 = -1 (* indicates complex number). G*(ω) has dimensions of Pa/μm and is related to the complex shear modulus of the cell by a geometric factor that depends on the cell-bead geometry (35). G(ω) accounts for the energy stored by the cell and G''(ω) for the energy dissipated within a cycle. The loss tangent, G''(ω)/G'(ω), indicates the balance between elastic and frictional stresses in the cell. A small loss tangent (<1) reflects solid-like elastic behavior, which enables the cell to maintain its shape in response to deformation. By contrast, a large loss tangent (>1) indicates the dominance of liquid-like cell behavior, which is required in functions such as crawling, spreading, or division in which the cell alters its shape and flows (18).

Modeling. G*(ω) data were fitted with the power-law structural damping equation (23, 26, 33) with the addition of a Newtonian viscous term (19)

$$G*(ω) = G_0(1 + jη)(\frac{ω}{ω_0})^α + jωμ$$

with

$$η = tan(απ/2)$$

where η is the hysteresivity or structural damping coefficient, α is the power-law exponent, and μ is a Newtonian viscous coefficient. G0 and ω0 are scaling factors for stiffness and frequency, respectively. In this model, G'(ω) increases with frequency following a power law with exponent α. At low frequencies, the Newtonian viscous term μω0 can be neglected. Therefore, G'(ω) increases proportionally to G'(ω) and η = G''(ω)/G'(ω). At high frequencies, G''(ω) is dominated by the viscous term and approaches the linear dependence of a Newtonian fluid. The soft glassy cell model introduced by Fabry and coworkers...
(19) assumes that $G^*(\omega)$ varies following Eqs. 4 and 5 and that $G_0$, $\omega_0$, and $\mu$ are constant within a given cell type. Accordingly, in the fitting, we took $\omega_0 = 2.5 \times 10^8$ rad/s, $G_0$ and $\mu$ were constrained to be constant among baseline and stretch conditions, but $\alpha$ was allowed to vary. Therefore, two values of the power-law exponent were obtained, corresponding to baseline and stretching conditions respectively. Fitting was carried out by nonlinear regression analysis (SigmaPlot; SPSS, Chicago, IL).

**Statistics.** Unless indicated otherwise, data are reported as means $\pm$ SE. Differences between results obtained with different experimental conditions were analyzed by the Student’s $t$-test for paired or unpaired observations, as appropriate. Specifically, comparisons of $G'$, $G''$, and $G'/G''$ between baseline and strain conditions were carried out with paired $t$-tests. Comparison of $G'$, $G''$, and $G'/G''$ between strain conditions [i.e., small strain (6.5%) and large strain (17%)] was carried out with an unpaired $t$-test. Statistical significance was assumed at $P < 0.05$.

**RESULTS**

The calibration of the stretching device is reported in Fig. 3. A linear relationship between pressure and strain was found with a maximum deformation of 20.5% for a vacuum pressure of 65 kPa. The error bars indicate the variability among wells ($n = 3$). As shown in Fig. 3B, the system was highly equibiaxial with discrepancies $<5\%$ between $\varepsilon_x$ and $\varepsilon_y$.

Figure 4 depicts how SS was transmitted to cells. LS closely matched SS over the range of deformations applied ($<5\%$ difference, $P > 0.2$). For the smallest strain amplitude (SS = 6.4%), no significant difference was observed between SS and the average CS (6.5%). However, for larger deformations, CS was significantly lower than the calibration value SS (SS = 17.3%, CS = 14.4%, $P < 0.01$). After the strain was held for 5 min, CS decreased further (12.6%) but nonsignificantly.

Figure 5A illustrates the bead displacement in response to an applied oscillatory torque of 1 Hz. A small phase lag was found between the applied torque and the resulting bead displacement, indicating viscoelastic cell behavior. Baseline values for $G^*$ (1 Hz) were $G' = 722 \pm 83$ Pa/µm and $G'' = 231 \pm 27$ Pa/µm with a loss tangent of $G''/G' = 0.336 \pm 0.012$. Stretching induced a significant rise in both $G'$ and $G''$ with maximum percent changes of 64 and 30%, respectively (Fig. 6). The increase in $G'$ was larger than that in $G''$, resulting in a slight but significant decrease in $G''/G'$. Changes in $G'$ and $G''$ were found to be significantly dependent on the stretching amplitude ($P < 0.01$ and $P < 0.05$, respectively). We observed little variations in $G'$, $G''$, and $G''/G'$ after holding the stretch for 5 min (data not shown). When the substrate was relaxed to its initial conformation, $G'$, $G''$, and $G''/G'$ recovered their baseline values ($P > 0.2$).

Disrupting the actin cytoskeleton with latrunculin A caused a $\sim$10-fold drop in cell stiffness (Fig. 7A). Baseline values of $G'$ and $G''$ were $74.1 \pm 17.2$ and $41.8 \pm 8.8$ Pa/µm, respectively, with a $G''/G'$ ratio of $0.638 \pm 0.019$. Application of an SS of 13.8% resulted in an actual CS of only 5.1% ($P < 0.001$) (Fig. 4). No significant changes in either $G'$ and $G''$ or $G''/G'$ were observed when stretching latrunculin A-pretreated cells (variation $<2.5\%$, $P > 0.4$; Fig. 7A).
Probing cell surface with acLDL-coated beads resulted in a ~20-fold decrease in baseline values of $G'$ and $G''$ compared with RGD-coated beads, yielding $G' = 29.1 \pm 3.5$ Pa/μm, $G'' = 6.1 \pm 0.9$ Pa/μm, and $G''/G'$ = 0.224 ± 0.009 (Fig. 7, B and C). After stretching (SS = 13.9%, CS = 10.6%), no significant change was observed in $G'$ ($P = 0.77$). By contrast, $G''$ exhibited a tendency to decrease with strain ($P = 0.07$), and $G''/G'$ decreased significantly with a relative change of ~8.8% ($P < 0.05$).

Increasing bead twisting frequency resulted in a fall in the bead displacement amplitude and in an increase in the phase lag between the applied torque and the resulting displacement, as illustrated by Fig. 5B. The dependence of $G^*$ with frequency under baseline conditions and for an SS = 14.1% ($CS = 12.5\%$) is plotted in Fig. 6. $G'$ increased with frequency following a power law as illustrated by the linear behavior in the log-log plot. At low frequencies, $G''$ was approximately threefold lower than $G'$ and exhibited a frequency dependence similar to that of $G'$. At higher frequencies, $G''$ exhibited a marked rise, crossing over $G'$ at ~100 Hz. Both $G'$ and $G''$ increased with stretching at all frequencies, remaining in agreement with the structural damping law. The rise in $G''$ was larger than that of $G'$, resulting in an average decrease in cell $\eta$ of 9%. Therefore, stretching induced not only an increase in cell stiffness but also a more solid-like elastic behavior of the cell. Fitting the structural damping equation to the whole dataset yielded $G_0 = 36.9 \times 10^3$ Pa/μm, $\mu = 2.8$ Pa·s/μm ($\gamma^2 > 0.98$) and resulted in a power-law exponent that decreased slightly with strain from $\alpha = 0.198$ to $\alpha = 0.173$.

**DISCUSSION**

We measured the $G^*$ of alveolar epithelial cells in culture subjected to equibiaxial distention with a novel experimental approach. We found that both the elastic and the frictional components of $G^*$ increased with strain and that the loss tangents decreased. The frequency dependence of $G'$ and $G''$, both before and after stretching, conformed to the structural damping equation. Disruption of the actin cytoskeleton inhibited the variation of $G^*$ with stretching. When cell viscoelasticity was probed with acLDL-coated beads, no significant stretch-induced response was observed in $G'$ and $G''$. Simultaneously with viscoelasticity measurements, we assessed the transmission of strain from the substrate to the cells. At low stretching amplitudes, SS was completely transmitted to the overlying cells, but, for large deformations, cells exhibited lower strain than the substrate.

The implemented experimental setup combines two techniques: equibiaxial substrate stretching and magnetic twisting cytometry with optical detection. The cell-stretching device includes features of previously described systems such as vacuum operation (5), equibiaxial strain by means of a frictionless loading post (40), and optical visualization of the sample with an inverted microscope (47, 50). All these features were required to allow simultaneous measurements of cell strain and cell rheology. Of the current microrheometry techniques, OMTC offered us unique advantages for this applica-
Indeed, it allowed us to selectively probe the mechanics of a large number of cells (typically 50 cells) in a fast single measurement. This contrasts with other techniques such as magnetic bead rheometry with sharp electromagnets (7), scanning force microscopy (39), optical tweezers (13), or microneedles (62), in which only a single cell is commonly probed. It is noteworthy that all the beads involved in one OMTC measurement are individually identified and processed with a multiple particle tracking system. Therefore, loosely bound beads or clusters that have been shown to bias the classical magnetic twisting cytometry measurements with magnetic detection are excluded (21). Given that the sample is visualized during the experiments, the same cells are probed before and after stretching. As the beads are firmly attached to the cell surface, measurements of $G^*$ are made at the same position of the cell body throughout experiments. Therefore, variability derived from the heterogeneity of the mechanical properties among the cell body (1) is avoided. Moreover, as the probe is carried by the sample and remains in a homogeneous magnetic field, fast experiments are possible. By contrast, the use of contact techniques such as scanning force microscopy or microneedles would require a slow and unreliable relocation of the probe after stretching. Finally, OMTC allows measurements in a broad bandwidth, obviating the need for corrections for the viscous drag on cantilevers or microneedles (3) or for the hysteresivity of soft iron cores used in magnetic bead rheometry with electromagnets.

We observed that equibiaxial stretching induced an increase in both the $G'$ and $G''$ of alveolar epithelial cells. By contrast,

![Fig. 7. Effect of stretching on $G'$ and $G''$ in cells pretreated with Lat A (1 μM, 30 min) (A) and in untreated cells probed with acetylated low-density lipoprotein (acLDL)-coated beads (B). For the sake of comparison, the data found in untreated cells probed with RGD-coated beads are also shown (C). $G'$ and $G''$ were measured under baseline conditions and after application of a stepwise substrate deformation. Changes between baseline and stretch were nonsignificant (NS) for Lat A-pretreated cells and for untreated cells probed with acLDL-coated beads. This contrasts with the significant changes observed in untreated cells probed with RGD-coated beads ($*$ and $**P < 0.05$ and $P < 0.01$, respectively).](#)

![Fig. 8. Frequency dependence of $G'$ (A) and $G''$ (B) under baseline conditions (filled symbols) and during application of a single stretch of $SS = 14.1\%$ (open symbols). Data are plotted as means ± SE. Solid lines are the fit of the structural damping equation (Eqs. 4 and 5) constraining the scaling factor for stiffness ($G_0$) and Newtonian viscous coefficient ($\mu$) to be constant among stretching conditions ($G_0 = 36.9 \times 10^3 \text{ Pa}/\mu\text{m}$ and $\mu = 2.8 \text{ Pa.s}/\mu\text{m}$) and allowing the power-law exponent ($\alpha$) to change with the challenge ($\alpha = 0.198$ before stretching and $\alpha = 0.173$ after stretching).](#)
no significant changes in $G'$ and $G''$ were observed in the step stretching measurements with previous disruption of cell cytoskeleton with latrunculin A, which prevents actin polymerization by sequestering actin monomers (14). We did not find stretch-induced changes in $G'$ and $G''$ either when cell surface was probed with acLDL-coated beads. AcLDL-coated beads bind to low-density lipoprotein receptors of the cell membrane, which do not play a role in cell adhesion and are not linked to the cytoskeleton (8, 56). These findings indicate that stretch-induced increases in $G'$ and $G''$ arise predominantly from the cytoskeleton. Interestingly, in measurements with acLDL-coated beads, $G''/G'$ decreased (9%), indicating a less liquid-like behavior of the cell surface. In this regard, it has been reported that cell membrane forms ruffles and invaginations that unfold in response to large deformations (53). The fall in $G''/G'$ we observed with acLDL-coated beads might reflect a reduction of the lipid reservoir of the cell surface.

We found that increases both in $G'$ and in $G''$ scaled roughly linearly with stretch (Fig. 6). $G'$ paralleled the rise of $G''$ but exhibited a smaller dependence on strain, resulting in a significant decrease in the loss tangent $G''/G'$. Therefore, stretching resulted not only in cell stiffening but also in an increase in the dominance of elastic over frictional stresses within the cell. Our results are consistent with earlier observations in endothelial cells stretched with lower stretching amplitude (<5%) (37). However, these authors only measured an apparent cell stiffness without taking into consideration the relative contribution of elastic and frictional stresses to cell stiffness. Moreover, in that study, the frequency dependence of the stretch-induced viscoelastic response was not investigated. Recent studies reported that modulating cell prestress by activation of cell contractile machinery with graded doses of contractile and relaxing agonists also induced a linear increase in $G'$ and $G''$ and a reduction in $G''/G'$ (44, 45). Thus increasing cytoskeletal tension with two entirely different procedures such as activation of molecular motors or cell distention resulted in a similar dynamic response. Together, these results suggest that cytoskeletal tension is a major determinant of both elastic and frictional processes in adherent cells. Several models of stress-supported structures have been proposed to describe the dependence of cell mechanical properties on cytoskeletal tension, including cortical membrane models (24, 25) and tensed cable nets (42, 55, 57). These models predict prestress-induced stiffening, but, in general, they neglect the contribution of frictional stresses. Recently, improvements in tensed cable net models in which elastic cables have been replaced by viscoelastic ones have been reported (11, 44). By using this approach, Stamenovic and coworkers (44) showed that the observed dependence of $G^*$ with prestress at a given frequency could be attributed to changes in spacing, reorientation, and elongation of cytoskeletal viscoelastic filaments.

The aforementioned models, however, fail to describe the cell dynamic response reported in the present paper. We found that, both before and after stretching, $G'$ and $G''$ increased following the structural damping law (Eqs. 4 and 5). When transformed into the time domain, this frequency dependence implies a continuous distribution of relaxation time constants. This contrasts with models of stress-supported structures composed of viscoelastic elements that exhibit a discrete number of time constants (43). Structural damping behavior has also been previously observed in several cell types (2, 19, 33, 38). Fabry and coworkers (19) reported that the scaling parameters of the structural damping law ($G_0$ and $\omega_0$) were constant within a given cell type and that only $\alpha$ varied in response to pharmacological challenges of the cytoskeleton. In this paper, we show that a mechanical challenge of the cytoskeleton leads to a similar mechanical behavior. Indeed, we were able to fit Eqs. 4 and 5 to the frequency dependence data allowing only the exponent of the power law to vary between baseline and stretch conditions ($\gamma^2 > 0.98$). In addition, as the ratio $G''/G'$ is entirely determined by $\alpha$, the structural damping expression predicts that cell stiffening is coupled to an increase in the solid-like behavior. Accordingly, Eqs. 4 and 5 predict that the increases found in $G'$ (29 and 64%) would be associated with decreases in $G''/G'$ of 6.9 and 13.0%. These predictions closely match the observed falls of 6.2 and 15.1% (Fig. 6).

To explain the empirical framework provided by the structural damping law (Eqs. 4 and 5), Fabry and coworkers (19) recently proposed that cells belong to the class of SGMs (41). Unlike the aforementioned models of stress-supported structures with a well-defined and deterministic geometry, SGMs are intrinsically disordered and exist in a metastable state far away from thermodynamic equilibrium. The mechanical behavior of SGMs arises from generic system features rather than from particular molecular properties. Structural elements are imagined to be agitated and rearranged by mutual interactions within a matrix (41). As such, the ability of the system to deform or flow is determined by the level of agitation of the elements within the matrix, which is characterized by the effective noise temperature ($x$) of the system defined as $x = \alpha + 1$. When $x = 1$, the elements of the matrix are trapped, providing the SGM with purely elastic behavior. In this case, the system deforms in response to the applied stress and recovers its original shape upon stress removal. By contrast, as $x$ increases toward $x = 2$, the elements become less and less trapped within the matrix. As a consequence, they can hop and reorganize, which confers fluid-like behavior to the SGM (18).

We observed that increasing cytoskeletal tension resulted in a significant decrease in $x$. According to the soft glassy hypothesis, such a decrease would be attributed to a reduction in matrix agitation. Although ATP-dependent mechanisms have been suggested (20), how matrix agitation is regulated within the cell remains an open question. Our results suggest that, whatever the source of agitation might be, it would be mediated by cytoskeletal prestress.

Alveolar epithelial cells subjected to stepwise deformations undergo a transient increase in intracellular calcium (59). This increase could trigger phosphorylation of the myosin light chain kinase and polymerization of actin, which, in turn, could result in a rise in cell stiffness. Therefore, passive and active stiffening mechanisms could coexist, i.e., the purely mechanical stiffening due to cytoskeletal distention and the stiffening due to actomyosin activation or actin polymerization. We observed that holding the stretch for 5 min did not result in further changes in $G^*$, suggesting that active mechanisms that could explain the observed increases in $G^*$ would be restricted to the first minute after stretching. Moreover, relaxing cell substrate to its initial conformation resulted in a recovery of the unstretched $G^*$ values, indicating that any stretch-induced actin polymerization or actomyosin activation would be reversible. Pourati and coworkers (37) provided evidence that stretch-induced stiffening response in endothelial cells is fun-
damentally passive. Indeed, they reported that clamping intracellular calcium and ATP to zero in permeabilized cells, as well as inhibiting cell oxidative metabolism, did not lead to any significant change in stretch-induced stiffening. Accordingly, although the active response cannot be completely ruled out, it is likely that the behavior observed in this study was primarily due to a purely mechanical increase in cytoskeletal tension.

Simultaneously with viscoelasticity measurements, we computed the strain undergone by the cells using a previously described method based on the assumption that the two-dimensional strain determined from bead displacements approximates the actual CS (6, 10, 30, 58). The published data comparing CC and SS do not provide conclusive results. Indeed, there is no agreement on whether actual CS coincides with or is smaller than the strain of the substrate (31). We observed that CS highly correlated with SS for small deformations (6.4%). However, for large stretching amplitudes (17.3%), the actual strain undergone by the cells was significantly lower than that of the substrate (14.3%). When the stretching step was held for 5 min, CS decreased further (12.6%). It has been speculated that these discrepancies between CS and SS could be due to the detachment of the cells or of the collagen coating layer from the substrate (59). However, we did not observe any significant difference between SS and strain measured from beads that were spaced several cell diameters from each other (LS). These results indicate that the collagen coating was firmly attached to the substrate and that the observed behavior was more likely due to a partial cell detachment. Data suggest that, for large stretching amplitudes, cell adhesive contacts with other cells and with the substrate are unable to withstand the increase in cytoskeletal tension, resulting in a partial cell detachment from the substrate. When cells were pretreated with latrunculin A, the actual CS dropped dramatically with respect to the SS. This behavior is attributable to the weakening of focal adhesion contacts induced by latrunculin A (9, 32). Our findings that cells at large strain may not match the strain of the substrate are also important when interpreting the results of cell stretching studies where only measured SS or manufacturer’s calibrations are employed.

Conclusion. We have shown that equibiaxial stretch induces an increase in both the storage and the loss moduli of human alveolar epithelial cells. The frequency dependence of the cell $G^*$ conformed to a structural damping power law whose exponent decreased with stretching. The increase in cell viscoelasticity induced by stretch was mostly inhibited by latrunculin A, showing that the actin cytoskeleton plays a major role in the cell mechanical response to stretch. These findings indicate that cytoskeletal prestress modulates both elastic and frictional properties of alveolar epithelial cells. Our results suggest that cell stiffening was paralleled by a partial cell detachment from the substrate at high strain amplitudes, which could indicate that cell adhesions did not withstand the increased tension. Stretch-induced stiffening might contribute to the disruption of the alveolar barrier in injured lungs subjected to mechanical ventilation by breaking the force balance at the cell-cell and cell-matrix adhesions.

ACKNOWLEDGMENTS

The authors thank Miguel Rodriguez for technical assistance.

GRANTS

This work was supported in part by Ministerio de Ciencia y Tecnología Grants SAF 2002-03616 and SAF 2003-01334, Ministerio de Sanidad y Consumo Grants Red GIRA-G03/063 and Red RESPIRA-C03/11, and National Heart, Lung, and Blood Institute Grant HL-65960.

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