Mechanical properties of alveolar epithelial cells in culture

Jorge C. Berrios, 1 Mark A. Schroeder, 1 and Rolf D. Hubmayr 1, 2
1 Thoracic Diseases Research Unit, Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, and 2 Department of Physiology and Biophysics, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Received 12 June 2000; accepted in final form 28 January 2001

Berrios, Jorge C., Mark A. Schroeder, and Rolf D. Hubmayr. Mechanical properties of alveolar epithelial cells in culture. J Appl Physiol 91: 65–73, 2001.—With the use of magnetic twisting cytometry, we characterized the mechanical properties of rat type II alveolar epithelial (ATII) cells in primary culture and examined whether the cells' state of differentiation and the application of deforming stresses influence their resistance to shape change. Cells were harvested from rat lungs as previously described (Dobbs LG. Am J Physiol Lung Cell Mol Physiol 258: L134–L147, 1990) and plated at a density of 1 × 10⁶ cells/cm² in fibronectin-coated 96 Remova wells, and their mechanical properties were measured 2–9 days later. We show 1) that ATII cells form much stronger bonds with RGD-coated beads than they do with albumin- or acetylated low-density lipoprotein-coated beads, 2) that RGD-mediated bonds seemingly “mature” during the first 60 min of bead contact, 3) that the apparent stiffness of ATII cells increases with days in culture, 4) that stiffness falls when the RGD-coated beads are intermittently oscillated at 0.3 Hz, and 5) that this fall cannot be attributed to exocytosis-related remodeling of the subcortical cytoskeleton. Although the mechanisms of force transfer between basement membrane, cytoskeleton, and plasma membrane of ATII cells remain to be resolved, such analyses undoubtedly require definition of the cell's mechanical properties. To our knowledge, the results presented here provide the first data on this topic.

mechanical stress; lung injury; cytoskeleton; magnetic twisting cytometry; cell adhesion

IN THE PAST DECADE, THE VENTILATORY management of patients with injured lungs has undergone a major paradigm shift (1, 11). Whereas the old paradigm considered the sole goal of mechanical ventilation to be the correction of gas exchange failure, the new paradigm seeks to provide “physical therapy” to an injured lung. This new paradigm embraces the hypothesis that rate and amplitude of lung deformation affect numerous lung cell metabolic functions and, consequently, modulate transcellular as well as intercellular fluid transport, mechanisms of inflammation, host defense, and wound repair. Because rat type II alveolar epithelial (ATII) cells are a popular culture model of the distal air space (30), a number of studies have sought to characterize cellular deformation responses using this system. Accordingly, it has been shown that mechanical distension of ATII cells in vitro causes calcium mobilization, stimulates surfactant secretion (46), and accelerates expression of type I cell characteristics (17).

Mechanical strain has been found to cause changes in cell morphology, alterations in cell cycle, and modifications of DNA synthesis and protein production in many different cell systems (14, 15, 21, 25, 47). Proposed mechanisms and molecular pathways governing mechanochemical signaling are numerous and include stretch-activated ion channels, intracellular calcium flux, G-protein-dependent and -independent kinase pathways, and the biopolymers of the cytoskeleton that physically link matrix-bound adhesion receptors and focal adhesion complexes (FACs) to the nucleus, chromosomes, and stress-responsive genes (4, 21, 25, 28). Because all these mechanisms operate in parallel, attributing deformation-related inflammatory signal transduction to any one pathway to the exclusion of the others is not justified. Although in some cells, stress-responsive elements have been identified and cloned, the site of mechanosensing in epithelial cells, and specifically in ATII cells, has not been established. Ingber (21, 22) and Maniotis et al. (28) consistently argued that the cytoskeleton, which is a prestressed network of molecular connections, provides a discrete path for mechanical signal transfer as well as a mechanism for producing integrated changes in cell and nuclear structure in response to mechanical stress. By actively regulating shape and stiffness, cells can adapt so that the same chemical or mechanical input produces a different output (cellular response), much like changing the tension of a string alters its pitch (23). It is in this context that we sought to characterize the mechanical properties of ATII cells in culture and to examine whether their state of differentiation and the application of deforming stresses influence their resistance to shape change.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
METHODS

Cell culture. ATII cells were harvested as described by Dobbs (8). Sprague-Dawley rats (300–400 g) were anesthetized by injection with pentobarbital sodium (70 mg/kg body wt ip), injected with 300 U of heparin, and euthanized by cutting the inferior vena cava. The trachea was cannulated with a 14-gauge catheter. After cannulation of the right ventricle, the lungs were perfused with 40 ml of solution II (in mM: 140 NaCl, 5 KCl, 2.5 NaHPO₄, 10 HEPES, 1.3 MgSO₄, and 2.0 CaCl₂). The lungs were lavaged multiple times with solution I (in mM: 140 NaCl, 5 KCl, 2.5 NaHPO₄, 10 HEPES, 6 glucose, and 0.2 EGTA) and once with elastase solution (30 elastase units/ml dissolved in solution II). Subsequently, the heart and lungs were excised en bloc, heart and trachea were removed, and the remaining lung tissue was incubated with the balance of elastase for 10 min and then minced with DNase (type I, 250 µg/ml). After the enzymatic activity was stopped with 5 ml of fetal calf serum (FCS; GIBCO BRL, Grand Island, NY), the mixture was incubated for 2 min in a 37°C shaking bath and then filtered once through gauze and twice through nylon mesh (150- and 15-µm pore size; Sefar-America, Kansas City, MO). The solution was then centrifuged for 10 min at 1,000 rpm, the supernatant was discarded, and the cells were reconstituted in Dulbecco’s modified Eagle’s medium (GIBCO BRL) without FCS. The cells were plated on IgG-coated petri dishes for 1 h. The media and nonadherent cells were aspirated and centrifuged for 10 min at 1,000 rpm. The supernatant was discarded, the cells were resuspended in 30 ml of Dulbecco’s modified Eagle’s medium with FCS, and cells were counted. Cells were then plated (1 × 10⁶ cells/well) to fibronectin-coated 96 microwells (Removawell, Dynatech Laboratories, Chantilly, VA).

Cell morphology. Cell projection imaging and measurements were performed by a modification of the protocol of Vlahakis and colleagues (43). Briefly, cells were lipid labeled with FM1-43 and immediately viewed with an argon ion laser confocal microscope (model FUX-SU, Olympus, Melville, NY) on an upright microscope (model BX50WI, Olympus) using a ×40 water immersion lens (Carl Zeiss, Thornwood, NY). The excitation and emission wavelengths were 488 and >515 nm, respectively. Surface area projection measurements were calculated with ANALYZE (Mayo Foundation, Rochester, MN). Data are presented as a percentage of total number of cells imaged vs. projected area.

Intercellular adhesion molecule-1 receptor expression. Intercellular adhesion molecule-1 (ICAM-1) receptor expression at the cell surface was measured after 1, 4, and 7 days in culture as previously described (2, 6, 19). Cells were grown on fibronectin-coated Lab-Tek chambered cover glass (Nalge Nunc, Naperville, IL) for 1 and 4 days. The cells were incubated at 37°C for 30 min with dansylated RGD peptide (Telios Pharmaceuticals, San Diego, CA), washed with PBS, and subsequently fixed for 10 min at 4°C with 2% paraformaldehyde. Cells were illuminated at a wavelength of 360 nm, viewed with an inverted microscope (model IX70-S1F2, Olympus), and imaged with a charge coupled device (model C4742-95, Hamamatsu, Bridgewater, NJ), and fluorescence was measured at an emission peak of 460 nm using the imaging package MetaMorph (Universal Imaging, West Chester, PA).

Magnetic twisting cytometry. Ferromagnetic (Fe₃O₄) microbeads (4.5 µm diameter, magnetic moment = 4 A m⁻² kg⁻¹) were coated with a synthetic RGD peptide (PepTide; Telios Pharmaceuticals) or acetylated low-density lipoprotein (LDL; Biomedical Technology, Stoughton, MA) at 50 µg/ml or incubated in bovine serum albumin (BSA). The RGD-, BSA-, or acetylated LDL-coated beads were dispersed in serum-free medium and added to each well at 20 µg/well (1–2 beads/cell) for 20 min. Unbound beads were washed away before magnetic twisting cytometry (MTC) measurements (20, 44). Cell wells were placed into the magnetic twisting cytometer and kept at 37°C. A 10-µs, 1,000-gauss homogeneous magnetic pulse was then applied to magnetize the beads in the horizontal direction. A flux-gate magnetometer (Foerster, Reutlingen, Germany) was used to measure the remnant magnetic field of the beads in the horizontal direction (Bᵣ). Values for Bᵣ are typically ~1 nT. A magnetic “twisting” field (<50 gauss), Hₛ, was applied in the vertical direction to twist the beads upward. Cell mechanical properties were derived from twisting torque and angular bead rotation (44, 45).

During twisting, the specific torque T (i.e., torque per unit bead volume) produced by a bead is as follows: T = c * H * cos(α), with the angular bead rotation (α) and the “bead constant” (c) expressed as torque per unit bead volume per gauss; c is determined by placing beads in a fluid of known viscosity and measuring the velocity of rotation during twisting. The bead’s specific magnetic saturation moment of 5 A m⁻² kg⁻¹ thus results in c of 4.1 dyn cm⁻² gauss⁻². In principle, when the beads are placed in an infinite elastic medium, they generate a shear stress that equals T. If the rotational strain α is known, the shear modulus G can be calculated as G = T/α; α can be calculated as follows: α = cos⁻¹(Bᵣ(t)/Bₒ), where Bₒ is the bead’s remnant magnetic field in the horizontal direction immediately after magnetization and Bᵣ(t) is the bead’s remnant magnetic field in the horizontal direction during twist.

MTC measurements were made on ATII cells 1) to assess the ligand specificity (RGD vs. BSA or acetylated LDL) of RGD-coated cells and 2) to characterize changes in apparent cell stiffness as a function of contact time between cells and RGD-coated beads (n = 6, 3) to measure stiffness of cells maintained in liquid culture for 2–9 days (n = 3–5/time point), 4) to characterize the effects of cyclic deformation on cell mechanics (n = 11), and 5) to assess the effects of the surfactant secretagogues ATP (100 µM, n = 5), terbutaline (100 µM, n = 4), and 12-O-tetradecanoylphorbol-13-acetate (TPA, 100 nM, n = 4) on cell mechanics.

Statistical methods. Values are means ± SD. Statistical comparisons between experimental conditions were made using analysis of variance or the Student’s t-test for unpaired and, when appropriate, paired observations. Statistical significance was assured at P < 0.05 with respect to a two-tailed probability distribution.
RESULTS

**ATII cells spread and assume a type I cell morphology with time in culture.** The average projected surface area of ATII cells increased from 206 μm² on day 2 to 1,073 μm² on day 5 and 811 μm² on day 12 (Fig. 1).

**ATII cells express an increasing number of ICAM-1 and integrin receptors with time in culture.** Figure 2 shows that adhesion receptor expression at the cell surface increased significantly between days 1 and 4. ICAM-1 receptor expression increased 66% during the first 4 days in culture and remained relatively unchanged thereafter. Similar results had been observed previously and were attributed to a loss of type II cell characteristics in favor of type I characteristics (6, 9). Over the same time span, binding of dansylated RGD peptide at the cell surface increased by 73%, indicative of increased integrin expression.

**ATII cells bind RGD-coated beads more avidly than they do albumin- or acetylated LDL-coated beads.** Figure 3 shows two MTC traces, one demonstrating the response of cell bound RGD-coated beads to applied stress and the other that of BSA-coated beads. Considering that the remnant magnetic field is inversely proportional to average bead rotation (strain), two observations are of note: 1) BSA-coated beads rotate further (~90° in this example) than RGD-coated beads when they are stressed (arrow); and 2) the remnant magnetic field of RGD-coated beads partially recovers after stress removal (at 80 s), whereas that of BSA-coated beads does not. For RGD-coated beads, this means that the deformed cell or, more specifically, its cytoskeleton exerts a force that reorients the beads in the direction of their prestressed baseline. However, the remnant field is not fully restored, suggesting that the cytoskeleton has been permanently deformed. The fact that BSA-coated beads rotate more or less freely suggests that albumin, in contrast to RGD, is not bound to cytoskeleton-associated receptors.

To further define the ligand specificity of MTC-based stiffness estimates, measurements were made using acetylated LDL-coated beads. This ligand binds to scavenger receptors that do not associate with the cytoskeleton (44). The results mirrored those obtained with BSA-coated beads, i.e., the derived stiffness values were 50–75% lower than when RGD-coated beads were used. Ligand-specific bead behavior implies that ATII cells in primary culture express apical integrin receptors that are capable of recruiting cytoskeleton proteins and form a FAC.

**Apparent stiffness of ATII cells increases with externally applied stress.** Figure 4 shows the average stress-strain relationships of ATII cells (n = 7 samples) 5–6 days after harvest. Stiffness is defined as the ratio of stress to strain, i.e., the inverse of the slope of the lines that connect each point to the origin. Because the relationship is nonlinear, it follows that the cytoskeleton stiffens when it is locally stressed. This behavior has been characterized as “strain hardening” (40). Accordingly, average cytoskeleton stiffness increased from 28 dyn/cm² at a stress of 11 dyn/cm² to 68 dyn/cm² at a stress of 59 dyn/cm². Twisting cytometry measurements have yielded nonlinear stress-strain relationships in many other cell types, an observation often cited in support of tensegrity-based models of the cell architecture (21, 39).

**Apparent stiffness of ATII cells increases the longer RGD-coated beads are in contact with the cell.** Apparent stiffness increases the longer RGD-coated beads remain in contact with the cells. In Fig. 5, apparent stiffness increased by 25 ± 10 dyn/cm² over the

*Fig. 1. Projected surface area of rat type II alveolar epithelial (ATII) cells after 1, 5, and 12 days in culture (n = 10–112 cells/time point).*

*Fig. 2. Cell surface adhesion receptor expression in ATII cells after 1, 4, 5, and 7 days in culture. Receptor expression correlates with relative light intensity, which was adjusted to 100 for day 1 samples. ICAM-1, intercellular adhesion molecule-1 (n = 4 samples/time point). RGD peptide signals integrin expression (n = 5–7 view fields/time point). *Significantly different from day 1 (P < 0.05).*

*Fig. 3. Twisting cytometry record of ATII cells after 48 h in culture. RGD-coated beads are compared with albumin-coated beads.*
course of 1 h. This observation is consistent with the ongoing recruitment of cytoskeletal elements to the focal adhesion sites between cells and beads. To address whether the time-dependent stiffening reflected a local reinforcement of bead binding consequent to repeated local deformations (5), we varied the number of measurements (twists) independent of bead contact time. For example, some of the 90-min data include measurements from wells that had been “twisted” only once, while others were twisted up to seven times starting at minute 30. Multivariate analysis showed that the number of measurements per well was not an independent determinant of apparent stiffness.

ATII cells become less deformable over time in culture. Figure 6 shows the average stiffness results of ATII cells between 2 and 9 days in culture. Two different analytic models were used to compute apparent cell stiffness; both yield qualitatively the same result, namely, ATII cells stiffen with time in culture. In the conventional model 1, angular rotation is computed relative to the bead orientation before the application of a twisting force (i.e., relative to the 20-s time point in Fig. 3). Model 1 ignores permanent deformation and, therefore, yields lower apparent stiffness values. In model 2, angular rotation is computed relative to the bead orientation after the application of a twisting force (i.e., relative to >120 s in Fig. 3). This model accounts for permanent deformation, insofar as only that part of the applied force that is stored in the cytoskeleton and responsible for the recoil of the beads to a new equilibrium position is considered in the stiffness calculation.

Apparent stiffness of ATII cells decreases with repeated deformation. Figure 7 shows a representative MTC tracing obtained from ATII cells with attached RGD-coated beads that were stressed at a rate of 10 cycles/min for 3 s at a time (i.e., at a duty cycle of 0.5). After every 10 cycles (once per minute), the magnetic moments of the beads were realigned in the direction of the magnetometer (10-μs discharges of 1,000 gauss, arrows), in effect restoring the remnant magnetic field to baseline. The twisting field of 80 gauss produced an average stress of 137 ± 3 dyn/cm², which varied systematically as a function of cycle and discharge number (see below).

Figure 8 shows that the apparent stiffness of ATII cells fell by 16 ± 14% (P < 0.001) in response to a 10-min cyclic deformation at 0.3 Hz. A small, but highly significant, fall in applied stress (0.9 ± 0.64 dyn·cm⁻²·min⁻¹, P < 0.01) was observed as well. The fall in applied stress is the consequence of permanent deformation accompanied by a gradual increase in the angular strain of the stressed beads. Recall that a bead with magnetic moment that is already aligned with the orthogonal twisting field (i.e., 90° relative to the magnetometer) is not stressed by it. Therefore, as beads increasingly align themselves with the twisting field, applied stress must fall. The reason for this alignment,

---

Fig. 4. Magnetic twisting cytometry-derived stress-strain relationship in ATII cells after 5–6 days in culture (n = 7). Bead ligand is RGD peptide.

Fig. 5. Average apparent ATII cell stiffness as a function of exposure duration to RGD ligand-coated beads (n = 6).

Fig. 6. Apparent stiffness of ATII cells between days 2 and 9 in culture (n = 3–5 samples/time point).

Fig. 7. Twisting cytometry record of ATII cells with RGD-bound beads that were exposed to a 3-s 80-gauss twisting field 10 times/min (cycles). Discharges of 1,000 gauss (downward arrows) were applied every minute to realign the bead magnetic moments with the magnetometer.
which is manifest as a temporal increase in angular strain (0.005 ± 0.004 rad/min, \( P < 0.01 \)), is local remodeling of the cytoskeleton at the bead attachment site or a more global change in cell deformability.

To ascertain that the fall in apparent stiffness with time and cumulative stress was not just a magnetic coil heating artifact, we also made standard stiffness measurements similar to those shown in Fig. 3 before and after exposure of the cells to cyclic deformation. During standard testing, the twisting field is applied for a full 1 min as opposed to only 3 s, leaving no doubt that the system has reached an elastic equilibrium. Figure 9 shows the individual and mean paired results from 10 wells. In every instance, we observed a decline in apparent cell stiffness averaging 12.6 ± 6% below baseline (\( P < 0.001 \)).

**Stress-induced decrease in apparent ATII cell stiffness reflects local cytoskeletal remodeling.** Rose and colleagues (34) showed that surfactant secretion requires remodeling of the subcortical cytoskeleton, as evident by depolymerization of F-actin in favor of G-actin. Because ATII cells are mechanosensitive and secrete surfactant in response to mechanical stimuli (46), it would seem reasonable to attribute the stress-related fall in apparent stiffness to this mechanism. For this hypothesis to be correct, we reasoned that secretagogues such as ATP, the \( \beta \)-agonist terbutaline, and phorbol ester should also cause a fall in apparent cell stiffness. The results of these experiments are summarized in Fig. 10. Neither ATP, terbutaline, nor TPA had any significant effects on the apparent stiffness of ATII cells. In light of this negative finding, we believe that local changes in the mechanical coupling between bead and cell (bead slippage or yielding) is a better explanation for the observed deformation response.

**DISCUSSION**

We have confirmed that primary ATII cells acquire certain type I cell characteristics, such as a flat shape and increased adhesion receptor expression, over time in culture (33). We have shown 1) that ATII cells form much stronger attachments with RGD-coated beads than they do with BSA- or acetylated LDL-coated beads, 2) that these attachments seemingly “mature” during the first 60 min of bead contact, 3) that the apparent stiffness of ATII cells increases, particularly between days 2 and 4 in culture, 4) that their stiffness falls when the RGD-coated beads are intermittently oscillated at 0.3 Hz, and 5) that this fall cannot be attributed to cytoskeletal remodeling associated with deformation-induced exocytosis. Before we discuss the biological implications of our findings, a discussion about MTC and the meaning of apparent cell stiffness is in order.

MTC is a cell mechanics measurement technique that was introduced in its present form by Wang and colleagues in 1993 (44). The technique rests on the observation that cells form an apical FAC with the beads so that bead rotation is impeded in some proportion to the cells’ resistance to a local shape change. Different mathematical analog models have been used to characterize the deformation resistance of cells and to compute apparent stiffness from bead torque and angular bead rotation (45). There continues to be uncertainty about the extent to which MTC probes the mechanical properties of the cell at large or mostly reflects the strength of the local bead coupling. This debate is heavily intertwined with arguments about
the usefulness of tensegrity as a model for understanding cell mechanics (18, 21). There is evidence in support of both sides of this argument. The observations that the apparent stiffness of ATII cells depends on the choice of ligand (Fig. 3) and increases as a function of bead contact time (Fig. 5) underscore the sensitivity of MTC on local bead adherence phenomena. On the other hand, the observations that bead manipulation at the cell surface can produce nuclear displacement and deformation (27, 28) and that contractile agonists increase the apparent stiffness of adherent human airway smooth muscle cells (20, 26) strongly argue in favor of some mechanical continuity between an apical FAC and more distant elements of the cytoskeleton.

Interpreted in the context of our study, we believe that changes in apparent ATII cell stiffness cannot be attributed to local cytoskeletal remodeling to the exclusion of a more global change in cytoskeletal mechanics and vice versa. We will return to this point in the discussion of the biological implications of our results.

For the most part, we chose to compute apparent stiffness without accounting for permanent deformation. As can be readily appreciated from Fig. 3, the remnant magnetic field, and hence the orientation of beads, is not fully restored after the beads have been strained (rotated). Although ignoring permanent deformation in the derivation of apparent stiffness biases the data toward low stiffness values, it does not bias temporal trends in cell mechanical properties. This is underscored in Fig. 6, in which two analytic approaches (models 1 and 2) are directly compared and yield qualitatively similar information: cells become less deformable over time in culture. This is consistent with findings in other cell systems, indicating that permanent deformation varies in a relatively fixed proportion with angular strain (3). Permanent deformation and apparent cell stiffness estimates are also biased by heterogenous bead binding (12). As pointed out by Fabry and colleagues (12), the nonlinear relationship between angular bead rotation (α) and the remnant magnetic field (Brem; see METHODS) gives much greater weight to weakly bound beads, which undergo large rotations, than to firmly bound beads, the magnetic moments of which remain closely aligned with the magnetometer. Hence, a change in apparent cell stiffness reflects a uniform change in the strength of apical bead binding, a shift in the distribution of binding properties across different cells, or a more global change in cytoskeletal mechanics. The latter might reflect a change in cytoskeletal prestress that is ultimately driven by the activation of motor proteins, a change in the connectivity between cytoskeletal elements, and/or a redistribution of loads carried by internal as opposed to external load-bearing elements.

**ATII cells bind RGD-coated beads more avidly than do albumin-coated beads.** Many of the integrins share an affinity to the RGD recognition sequence that can be found in many extracellular matrix proteins such as fibronectin, laminin, and collagen (35). It should come as no surprise that albumin- and acetylated LDL-coated beads associated less avidly with ATII cells than beads coated with a matrix recognition sequence, namely, RGD (35). Similar observations, namely ligand-specific stiffness estimates, have been made in other systems (3, 44). The difference in the mechanical interactions of the cell with RGD- as opposed to BSA-coated beads would imply that ATII cells do not possess albumin receptors (36) and/or that albumin receptors do not strongly associate with the cytoskeleton. This is akin to the association of acetylated LDL-coated beads and scavenger receptors observed in other cell systems (44). Results of an albumin competition assay (data not shown) suggest that ATII cells have no albumin receptors. More importantly, the cell mechanical responses to RGD-coated beads suggest that ATII cells express apical integrins as early as 2 days after cell harvest in liquid culture. This interpretation of our MTC responses (Fig. 3) is consistent with studies in which the expression of adhesion proteins by ATII cells in liquid culture have been characterized (6, 24) and our own findings with fluorescent-tagged RGD sequences (Fig. 2). ATII cells lose many type II features soon after cell harvest and begin expressing type I characteristics, including ICAM-1, a member of the immunoglobulin superfamily (38), and αvβ5 integrins. Accordingly, the adhesion of ATII cells to provisional matrix can be inhibited with synthetic RGD peptide (24).

**Bonds between RGD-coated beads and ATII cells “mature” during the first 60 min of bead contact.** Figure 5 shows that ATII cells appear to stiffen during the first 90 min of contact with RGD-coated beads. This observation suggests that the fraction of strongly bound beads increased over this time period, that FACs continued to mature and recruited additional cytoskeletal elements to the bead attachment site, and/or that the exposure to a provisional matrix over the apical cell surface caused a more widespread, global reorganization of the cytoskeleton. Magnetic as well as latex microbeads coated with fibronectin or RGD peptide have been shown to induce local FAC formation as early as 15 min after bead application in virtually all cell systems studied (16, 32). FACs are mechanosensitive structures and are intimately involved in bidirectional integrin-mediated signaling (5, 37, 48). Choquet and colleagues (5) observed that human fibroblasts “probe” the rigidity of their surrounding matrix and that they modulate and adapt both strength and molecular composition of their adhesion sites accordingly. To examine whether deformation-induced FAC reinforcement played a part in the apparent stiffening response of ATII cells, we varied the number of “twists” (i.e., stiffness measurements) independent of bead contact time. Because we could not demonstrate a mechanosensitive reinforcement effect, we speculate that either the cells were not strained long and often enough for reinforcement to take place or epithelial cells differ from mesenchymal cells in this respect. The localized application of matrix protein to the apical surface of baby hamster kidney cells has been shown to alter their adherence and spreading characteristics, implying a downstream effect on dis-
tant (basal) adhesion mechanisms (7). This observation is in keeping with a more global effect of RGD on cytoskeletal organization and possibly on cell prestress. Because there is considerable controversy about the nature of load-bearing elements and, hence, about the stress distribution within cells (18, 22), one must be careful in attributing changes in apparent cell stiffness to local remodeling as opposed to global changes in cytoskeletal tension.

**ATII cells become less deformable over time in culture.** It has been shown that ATII cells lose their type II characteristics and acquire those of type I cells when they are grown in vitro. We chose two previously described cell markers to demonstrate cell differentiation in our culture system: cell morphology and ICAM-1 expression. Type II cells in vivo are cuboidal in shape, whereas type I cells appear more spread and flat. Cell projection analysis revealed that ATII cells’ average area increased almost fourfold from projection analysis revealed that ATII cells’ average area increased almost fourfold from days 2 to 12 in culture. In vivo ATII cells express minimal amounts of ICAM-1, as opposed to type I cells. In our system, freshly harvested ATII cells showed a small amount of ICAM-1 expression, but by day 4 in culture this had increased by 66% and remained mostly unchanged thereafter. We also discovered a novel finding, that integrin expression increases in ATII cells with time in culture as well.

Figure 6 indicates that freshly harvested cells grown over 48 h in culture appear less stiff than confluent monolayers (day 4 in culture and beyond). Stabilization from cell-cell contact via E-cadherins and tight junctions had been demonstrated in other epithelial cell systems (3). There are a number of additional likely reasons for the temporal change in ATII cell mechanical properties. In virtually all cell systems in which this has been evaluated, flat, spread cells appear stiffer than round, cuboidal cells (20, 21, 45). Although proponents of tensegrity as a model for understanding cell mechanical behavior predict a shape dependence of prestress and, therefore, of cell stiffness (39), the observation might simply reflect the proximity of the bead and its FAC to basal stress fibers and/or FACs between cell and substratum. In a continuum mechanics analysis, this observation would be characterized as a boundary effect. Finally, the greater number of adhesion receptors, specifically integrins, at the surface of differentiating ATII cells might promote a more robust FAC formation and thereby strengthen the mechanical coupling between cells and beads.

**Apparent stiffness of ATII cells decreases with repeated deformation.** The experiments summarized in Figs. 8 and 9 provide unequivocal evidence that a 10-min application of an intermittent local stress at a frequency of 0.3 Hz reduces the apparent stiffness of ATII cells. In keeping with our view of MTC-derived mechanics indexes, we thought to relate the changes in apparent cell stiffness to evidence of local or global cytoskeletal remodeling. The observation reminded us that ATII cells are mechanosensitive, that is, ATII cells release surfactant in response to deformation (46), and that surfactant exocytosis is associated with remodel-

ing of the subcortical cytoskeleton (34). Specifically, Rose and colleagues (34) showed in the same ATII cell culture model that the surfactant secretagogues ATP, TPA, and terbutaline induce subcortical actin depolymerization, thereby facilitating vesicular transport to the plasma membrane. Although we were able to confirm that TPA induces surfactant secretion (data not shown) as Rose and colleagues had done, we did not find a corresponding change in cell mechanical properties (Fig. 10). Indeed, none of the three pharmacologically surfactant secretagogues produced a measurable change in apparent cell stiffness. This suggests that the measured stiffness might not reflect the stiffness of the subcortical F-actin network and that it is not possible to attribute deformation-induced changes in apparent cell stiffness to cytoskeletal remodeling associated with exocytosis.

In a recent publication on human airway smooth muscle cells in culture, Maksym and colleagues (26) demonstrated relative stability in cell mechanical properties during the application of an oscillatory stress. However, our experiment on ATII cells differed from that of Maksym and colleagues in several important ways. They applied sinusoidal oscillations between 0.05 and 0.5 Hz, whereas we rotated the beads in one direction and allowed them to recoil passively. Because of permanent deformation, beads would typically recoil within 30–40° of their original orientation. We remagnetized the beads every minute to realign their magnetic moments with the magnetometer (see arrows corresponding to discharges in Fig. 7). As a result, a significant fraction of beads underwent rotations in excess of 360° from their original orientation over the course of a 10-min experiment. We put forth the hypothesis that such large angular rotations relative to baseline (time 0) promote FAC remodeling and bead slippage. From scanning electron microscopy imaging, we know that on average only the basal aspect of a bead is in contact with the apex of the cell (data not shown). Consequently, the application of a twisting torque brings unoccupied RGD molecules of the free bead surface in contact with integrins that reside in the plasma membrane, while at the same time lifting and pulling the FAC attached to the bead base out of the plane with the original cell border. As a result, we speculate that beads continuously formed new FACs over their free surface while resolving “more mature FACs” originally situated at the bead base. Given the observations of Fig. 5 that suggested to us that new FACs are “weaker” than established FACs, we postulate that strain-induced bead slippage accounts for the fall in apparent stiffness of ATII cells in our experiment.

**Biological implications and concluding remarks.** We set out to characterize the mechanical properties of a popular culture model of the distal air space. Our motivation was our interest in ventilator-induced lung injury and thus the biological responses of this model to deformation. We had recently shown that primary rat ATII cells release tumor necrosis factor-α when intermittently strained (31) and speculated that non-
lethal plasma membrane wounding (plasma membrane stress failure) is an important mechanotransduction event for the upregulation of proinflammatory genes (42). It would stand to reason that the probability of plasma membrane wounding is in some way related to the cells’ resistance to a shape change, which we now know changes with time, shape, and differentiation of these cells in culture. Consistent with these ideas, Tschumperlin and Margulies (41) reported that recently harvested ATII cells are more likely to take up ethidium homodimer (a marker of membrane integrity) in response to a stretch than cells that had grown to confluence for 5 days in culture. Because for a given substratum strain the load on force-bearing elements of the cell varies with the cell’s resistance to shape change, we postulate that the changes in apparent ATII cell mechanical properties will scale with other deformation responses, such as lipid trafficking and cytokine release, as well. Although the mechanisms of force transfer between basement membrane, cytoskeleton, and plasma membrane of ATII cells remain to be resolved, such analyses undoubtedly require definition of the cell’s mechanical properties. To our knowledge, the results presented here provide the first data on this topic.

The authors thank B. J. Walters and P. J. Zollman for technical support and L. L. Oeltjenbruns for manuscript preparation. This work was supported by National Heart, Lung, and Blood Institute Grant HL-63178.

REFERENCES