Is cytoskeletal tension a major determinant of cell deformability in adherent endothelial cells?

JACOB POURATI,1 ANDREW MANIOTIS,2 DAVID SPIEGEL,1 JONATHAN L. SCHAFFER,3 JAMES P. BUTLER,1 JEFFREY J. FREDBERG,1 DONALD E. INGBER,2 DIMITRIJE STAMENOVIĆ,4 AND NING WANG1

1Physiology Program, Harvard School of Public Health and 2Departments of Pathology and Surgery, Children's Hospital and Harvard Medical School, Boston 02115; and 3Departments of Orthopedic Surgery, Brigham and Women's Hospital, Children's Hospital, and Harvard Medical School and 4Department of Biomedical Engineering, Boston University, Boston, Massachusetts 02215

Pourati, Jacob, Andrew Maniotis, David Spiegel, Jonathan L. Schaffer, James P. Butler, Jeffrey J. Fredberg, Donald E. Ingber, Dimitrijie Stamenovic, and Ning Wang. Is cytoskeletal tension a major determinant of cell deformability in adherent endothelial cells? Am. J. Physiol. Cell Physiol. 43: C1283–C1289, 1998.—We tested the hypothesis that mechanical tension in the cytoskeleton (CSK) is a major determinant of cell deformability. To confirm that tension was present in adherent endothelial cells, we either cut or detached them from their basal surface by a microneedle. After cutting or detachment, the cells rapidly retracted. This retraction was prevented, however, if the CSK actin lattice was disrupted by cytochalasin D (Cyto D). These results confirmed that there was preexisting CSK tension in these cells and that the actin lattice was a primary stress-bearing component of the CSK. Second, to determine the extent to which that preexisting CSK tension could alter cell deformability, we developed a stretchable cell culture membrane system to impose a rapid mechanical distension (and presumably a rapid increase in CSK tension) on adherent endothelial cells. Altered cell deformability was quantitated as the shear stiffness measured by magnetic twisting cytometry. When membrane strain increased 2.5 or 5%, the cell stiffness increased 15 and 30%, respectively. Disruption of actin lattice with Cyto D abolished this stretch-induced increase in stiffness, demonstrating that the increased stiffness depended on the integrity of the actin CSK. Permeabilizing the cells with saponin and washing away ATP and Ca2+ did not inhibit the stretch-induced stiffening of the cell. These results suggest that the stretch-induced stiffening was primarily due to the direct mechanical changes in the forces distending the CSK but not to ATP- or Ca2+-dependent processes. Taken together, these results suggest preexisting CSK tension is a major determinant of cell deformability in adherent endothelial cells.

is a basic determinant of cell shape and function in adherent cells (12–14, 18, 27, 29). According to this hypothesis, the level of preexisting mechanical tension (or initial tension, defined as tension residing in CSK before mechanical measurements) is predicted to be a major determinant of cell deformability: the higher the initial tension, the stiffer the cell would be. Although it has long been known that several cell types are under tension (1, 2, 10, 16), it has not been shown that this tension plays a role in regulating cell deformability. The main goal of this study was to show that the CSK tension influences cellular resistance to shape distortion in a stretch-dependent manner in adherent endothelial cells.

We tested the hypothesis in two parts. First, we confirmed the presence of initial tension in living adherent endothelial cells by rapidly cutting them with a microneedle or by dislodging focal adhesions. The rationale was that if the CSK is initially tensed, then the cell would rapidly retract after the cut, as would a tensed violin string. We found that the cell did retract rapidly after cutting. Second, we assessed, indirectly, the effects of changes in CSK initial tension on CSK stiffness. To do this, we modified the stretchable membrane system of Schaffer et al. (23) so that it could fit into a magnetic twisting cytometry (MTC) device. Our rationale was that a rapid uniform distension of the substrate to which the cell is adherent would increase the CSK distension and thus increase the tension in the CSK lattice. The hypothesis predicts that this should manifest itself by an immediate increase in CSK stiffness. We found that, after a rapid stretch, the cells did exhibit a stretch-dependent increase in CSK stiffness. This finding is consistent with the notion that the CSK is initially tensed and that this tension is a major determinant of cell deformability.

MATERIALS AND METHODS

Cell cutting. Endothelial cells were plated sparsely in serum-free medium on coverslips coated with high densities of fibronectin (500 ng/well) permissive to sustained cell attachment for 4 h. A coverslip was then placed into a 35-mm-diameter petri dish containing fresh medium. A layer of mineral oil was layered over the medium to maintain pH. Then the petri dish was placed on an Omega RTD 0.1°-stable stage heating ring coupled to a Nikon Diaphot inverted microscope. Images were obtained with a Citron videocamera and recorded on a GYYRE video recorder. Microneedles were pulled with a Sutter micropipette puller, adjusted to produce long tips of 1- to 5-µm diameter, with a length of 40–100 µm. To determine whether these cells carry an initial tension, they were cut by a microneedle across the cytoplasm. The ensuing change of cell shape was quantitated.

Cell-stretching system. A schematic diagram of the cell-stretching system is shown in Fig. 1. A 76-µm-thick membr...
brane of special formulation silicone elastomer (Dow Corning, Midland, MI) was tightly clamped onto a bottomless 96-well plate by pushing a clamp over the well to prestretch the membrane. A 4.4-mm-diameter platen was placed at the bottom of a plastic vial, and the membrane well was placed on top of platen. A threaded rod was screwed down to push membrane well downward through a spacer. Because platen was stationary, downward movement of membrane well results in upward stretching of membrane on which cells are attached. Whole stretching system was placed into magnetic twisting cytometer.

Calibration of cell-stretching system. Dots were drawn on the prestretched membrane with a fine-tipped pen. The positions of dots at different states of stretching were observed with a dissecting microscope and recorded with a digital charge-coupled device camera connected to a computer with Photometrics graphics software. Stretch was calculated as the ratio of the postdisplaced dot relative positions to the predisplaced relative dot positions (23). Strain was defined as stretch minus 1. There was a positive, but nonlinear relation between the rotation of the threaded platen against a platform (i.e., upward movement of the platen) and the strain of the membrane (Fig. 2). To further determine whether the stretching of the membrane was uniform, strains in two orthogonal directions (X and Y) were measured. We found that the membrane stretching was uniform up to 5% strain of the membrane with a diameter of 4.4 mm (Fig. 3). The Young's modulus of the membrane was found to be 2.7 × 10^8 dyn/cm^2. There was no breakage, leakage, or buckling of the membrane after repeated stretches.

Cell cultures for stretching. Bovine capillary endothelial cells were cultured to confluence, serum deprived, trypsin-

Fig. 1. Schematic of cell-stretching system.

Fig. 2. Calibration of rotation of platen (upward movement of platen) and actual strains of membrane. Strain is defined as stretch minus 1. Stretch is defined as ratio of distance between 2 dots after distension to distance between same 2 dots before distension. •, Strain measured in X direction; ■, strain measured in Y direction. Means ± SE; n = 4 wells.

Fig. 3. Calibration of biaxial strain of membrane during stretching. Fine dots were drawn in black ink in 2 mutually orthogonal directions (X and Y). Positions of dots before and after stretching membrane were recorded with a digital camera connected to a 386 Gateway computer and Photometrics graphics software. X and Y strains represent dots close to edge of flat surface of membrane. Dots close to center of membrane displayed similar results (not shown). Means ± SE; n = 4 wells.
ized, and plated in defined medium overnight on membrane
dwells that were precoated with human serum fibronectin
(Cappel) at 2 μg/well (30). To ensure that cells were plated
only onto the part of the membrane which was uniformly
stretched (4.4-mm diam), a rubber tube of 4.4 mm ID was
inserted onto the well just before the cells were plated at
20,000/well. This rubber dam was removed before twisting
experiments. The cells were plated 4–10 h and were subcon-
fluent during the whole experiments.

In studies analyzing the role of membrane integrity and
ATP-dependent biochemical processes, cells were permeabi-
ized with saponin as previously described (25, 30). Briefly,
cells were cultured overnight onto the membrane well. They
were washed once in a CSK stabilization buffer (50 mM KCl,
10 mM imidazole, 1 mM EGTA, 1 mM MgSO4, 0.5 mM
dithioreitol, 5 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl
fluoride, and 20 mM PIPES, pH 6.5). Cells were then incu-
bated in the same buffer containing saponin (25 μg/ml; Sigma,
St. Louis, MO) for 8 min at 37°C, and mechanical properties
were measured before and after stretching the membrane.

MTC. The mechanical properties were quantitated using
MTC as described previously (29–31). Ferromagnetic beads
(4.5-μm diam, provided by Dr. W. Moller, Germany) were
coated with Arg-Gly-Asp peptides, which bind specifically to
 integrin receptors. These beads were added to each mem-
brane well at 20 μg/well (avg 2 beads/cell) for 15 min. The well
was then washed once with 1% BSA-DMEM to remove
unbound beads. An initial magnetic stress (torque/bead vol-
ume) of 60 dyn/cm² was applied to the cells through the beads
and held for 60 s. Corresponding changes in the angular
strain (a form of shear strain) of the beads were measured.
Stiffness was defined as the ratio of shear stress to shear
strain. The well membrane was then rapidly stretched for 10
s, the same torque was applied, and the mechanical measure-
ments were repeated.

RESULTS

Initial tension is present in living adherent cells. To
confirm whether living adherent endothelial cells carry
initial tension, we observed shape changes after a cut
by a microneedle attached to a micromanipulator. We
reasoned that initial tension in the CSK, if any, must be
in static mechanical equilibrium (9), but when the cell
is cut, the static equilibrium is upset and a rapid
deformation must ensue. The results showed that the
initial separation between two parts of the cell in-
creased rapidly after the cut, like a recoil of an elastic
material. The rapid retraction period generally lasted
<10 s, followed by a slow retraction period that oc-
curred over the course of minutes. However, both the
fast and the slow phase of the retractions were com-
pletely prevented when the cell was pretreated with
cytchalasin D (Cyto D, 1 μg/ml) for 30 min (Fig. 4A).
The rapid retraction might be attributable to the
sudden release of the initial tension and the ensuing
passive mechanical creep of the associated mechanical
structures; however, an alternative explanation is that
placements observed after the cut were an active
response to cell injury. To minimize cell injury, we used
a method that was developed by Albrecht-Buehler (1).
We placed a microneedle underneath the basal surface
of the cell and rapidly dislodged focal adhesions under
long processes extending from the cell body. As in the
cutting experiment, we observed that the long exten-
sions retracted rapidly (<10 s) toward the cell center
(n = 20 cells) when the focal adhesion was dislodged.
This retraction was inhibited by pretreatment with
Cyto D (1 μg/ml for 30 min; n = 20 cells; Fig. 4B).
Mechanical distension alters cell stiffness. Increasing
the distension of the membrane substrate increased
cell stiffness: 2.5% membrane strain resulted in ~15% in-
crease in the stiffness (P < 0.05), and 5% membrane
strain resulted in ~30% increase in the stiffness (P <
0.05 compared with 2.5% strain; P < 0.01 compared
with control; Fig. 5). Stretching the cells and holding
the stretch for 3 min at 5% strain increased the stiffness
by another 10% (data not shown).

To confirm that the CSK actin lattice contributed to
the observed stretch-induced stiffening, adherent cells
were stretched before and after addition of Cyto D,
which disrupts the actin lattice. Addition of Cyto D (1
μg/ml for 30 min) resulted in a 40% reduction in
stiffness from the control (Fig. 6). Cyto D also com-
pletely prevented the effects of the stretch on cells.
These data demonstrate that the stretch-induced stiff-
ening response required the presence of the microfila-
mament lattice.

To determine whether the stiffening response de-
pendent on membrane integrity, ATP, or Ca²⁺, the
stiffness was measured when the cells were permeabi-
lized with saponin (25 μg/ml) for 8 min; intracellular
ATP and Ca²⁺ were then clamped at zero. In intact
cells, a 5% stretch increased the stiffness by ~30% (P <
0.005). Addition of saponin in the absence of stretch
increased the stiffness by 5% (P < 0.01), consistent with
our earlier results (30). A 5% stretch in the presence of
saponin resulted in a 25% increase in stiffness (P <
0.05). There was no significant difference in stiffness in
the absence or presence of saponin for stretched cells
(Fig. 7). Furthermore, a 5% strain in cells pretreated
with the inhibitor of oxidative metabolism 2,4-dinitro-
phenol (DNP, 1 mM for 15 min) still induced >20% in-
crease in stiffness (Fig. 8), demonstrating that DNP
did not have an effect in inhibiting the stiffening response.
Therefore stretch-induced increases in cell stiffness
appear to be not dependent on chemical changes but
dependent on mechanical changes.

DISCUSSION

The most significant finding of this study is that a
rapid stretch of adherent endothelial cells resulted in a
prompt increase in CSK stiffness. In addition, the cell
cutting and dislodging results confirmed earlier find-
ings that adherent cells are initially tensed. Both re-
ponses were inhibited by disruption of the actin lattice,
suggesting that the presence of an intact actin lattice
is required for stress transmission throughout the cell.

Cell cutting might cause cell injury that could lead to
release of molecules, such as Ca²⁺, which in turn might
induce cell retraction. However, we also observed cell
retraction when long processes of the cell were dis-
lodged from the substrate. This detachment technique
probably caused much less cell injury but yielded
essentially equivalent findings. Furthermore, cell re-
traction after the cut or detachment was completely
prevented with Cyto D pretreatment, which might not inhibit Ca\(^{2+}\) release. Although we cannot entirely rule out other interpretations, the results presented here are consistent with the interpretation that preexisting tension was present in the living adherent endothelial cells that we studied.

Despite the fact that the membrane was stretched in a short time interval (<10 s) and stiffness was measured within 70 s, there remain the possibilities that the CSK might have remodeled in response to stretch and affected CSK stiffness. For instance, intracellular K\(^+\) and Ca\(^{2+}\) have been shown to be activated within seconds after mechanical deformation (5). Other intracellular responses, such as transient elevation of inositol lipids, could also happen on the order of 30 s. Although we cannot exclude these possibilities, we performed tests that showed that stretch-induced stiffening occurred in ATP- and Ca\(^{2+}\)-free permeabilized cells (Fig. 7). Furthermore, stretch-induced stiffening also persisted in intact cells in which oxidative metabolism was inhibited. In addition, this stretch-induced stiffening was prevented after cells were treated with Cyto D, demonstrating that this response was dependent on the presence of intact actin lattice. Therefore, although other mechanisms cannot be ruled out, we favor the interpretation that stretch-induced stiffening response was primarily due to increase in the distending forces within the CSK.

These findings extend previous studies showing that initial tension may play an important role in regulating cell deformability (i.e., cell shear stiffness). For example, it has been shown that highly spread endothelial cells are stiffer than less spread cells (30, 31), but there may be many processes besides CSK tension, such as actin polymerization and CSK remodeling, which could have influenced CSK stiffness. In contrast, the study presented here minimized the effects of these processes. In another study, CSK tension in airway...
Fig. 5. Stretch-induced stiffening depends on degree of stretching. Endothelial cells were plated on membrane wells overnight in defined medium in absence of growth factors or serum. Arg-Gly-Asp-coated beads were bound to adherent, spread, and subconfluent cells for 15 min and unbound beads were washed away. An initial stress of 60 dyn/cm² was applied. Membrane was rapidly stretched for 10 s; same stress was applied and stiffness was measured again. Different wells were used for 2.5% strain and 5% strain. Means ± SE; n = 6 wells for 2.5% strain; n = 8 wells for 5% strain. A dozen other experiments showed similar results.

Fig. 6. Effects of microfilament lattice disruption on stretch-induced response. Stiffness was measured in adherent endothelial cells; same cells were then rapidly stretched at 5% for 10 s, and stiffness was measured again. Cyto D (1 µg/ml for 30 min) was added to same cells to disrupt microfilament lattice and stiffness was measured again before and after another 5% strain (S). It appears that disruption of microfilament lattice abolished stretch-induced stiffening response. Means ± SE; n = 4 wells. Two other independent experiments showed similar results.

Fig. 7. Effects of membrane permeabilization on stretch-induced response. Adherent endothelial cells were stretched at 5% strain and stiffness was measured before and after stretch. Then saponin (25 µg/ml for 8 min) was added to same cells to permeabilize cells, and ATP and Ca²⁺ were washed away. Same cells were twisted again before and after permeabilization. Note that removal of ATP and Ca²⁺ did not have any significant effects on stretch-induced stiffening response. Means ± SE; n = 6 wells. An independent experiment showed similar results.

Fig. 8. Effects of oxidative metabolism inhibition on stretch-induced response. Adherent endothelial cells were treated with 2,4-dinitrophenol (1 mM) for 15 min before experiments. A rapid 5% stretch was applied to cells and stiffness was measured. Means ± SE; n = 8 wells. An independent experiment showed similar results.
smooth muscle cells has been altered at a fixed state of spreading by adding bronchoconstrictors or bronchodilators (11); it was found that CSK stiffness increases in cells treated with bronchoconstrictors and decreases in cells treated with bronchodilators over time scales of <1 min. These changes in CSK stiffness are thought to be mediated through activation or deactivation of actomyosin apparatus, thus changing the active tension in the CSK. However, addition of contractile agonists to the smooth muscle cells may also trigger processes, such as phosphorylation of talin and paxillin (19), which in turn may affect CSK stiffness by altering focal adhesion complexes. CSK stiffness has also been increased by overexpression of myosin light-chain kinase in fibroblasts (6). However, overexpression of myosin light-chain kinase might activate processes other than actomyosin cycling, which in turn could affect the architecture and mechanics of the CSK. Moreover, in all these previous studies, the passive components of the CSK tension had not been manipulated. In contrast, by applying rapid mechanical stretches to the cells, we were able to minimize the time available for active cellular responses.

It appears that the results presented here are not easily explained by linear continuum models of cellular mechanics. Models suggested in the literature include linear elastic or viscoelastic half-space models (22, 28), models in which continuum mechanical properties of the CSK are deduced from the mechanical properties of individual actin filaments (20), and models depicting the adherent cell as a viscous, viscoelastic, or elastic cytoplasm enclosed by an elastic membrane (9, 21, 24).

Given that stretching was rapid, cell volume would not change very much. Accordingly, a 5% strain (i.e., an ~10% increase in cell basal surface area) would result in an ~10% reduction in cell height. Any linear continuum model would predict, at most, a 10% increase in stiffness. This is so because force transmission between the bead and the substratum would take place mainly through the portion of the cell underneath the bead. In the case of the model of viscous cytoplasm enclosed by linearly elastic membrane, a 10% decrease in cell height would produce an even smaller fractional increase in stiffness. However, we found that a 5% stretch produced a disproportionate (20–30%) increase in CSK stiffness (Figs. 5–7).

If linear continuum models of cellular mechanics are inappropriate to explain our observations in adherent cells, then either a nonlinear continuum model or an approach that emphasizes the discrete, as opposed to the continuous, nature of the CSK microstructure needs to be used. If the former, then the elastic properties of the continuum would have to be assigned on an ad hoc basis to account for the dependence of cell stiffness on cell distension reported here. If the latter, in contrast, nonlinear behavior of the CSK may be an intrinsic property conferred by the microstructural architecture (4, 27). In that case, nonlinearity of the individual discrete elements is not precluded, but it is not necessary to postulate such nonlinearity to account for the essential features of the data. Interestingly, discrete but nonpretensed models of percolation that analyze phase transitions and connectivity within networks (8) do not appear to be consistent with our results.

In summary, we have presented evidence that stretching adherent endothelial cells on an elastic membrane results in an increase in CSK stiffness. This is likely to be the result of an increase in passive CSK tension due to increased cell distension. Therefore distending stress of the CSK appears to be a key determinant of cellular deformability.

This work was supported by National Institutes of Health Grants HL-33009, CA-45548, HL-56398, and AR-41352.

Address for reprint requests: N. Wang, Physiology Program, Harvard School of Public Health, 665 Huntington Ave., Boston, MA 02115.

Received 1 October 1997; accepted in final form 11 February 1998.

REFERENCES


