Contribution of actin filaments and microtubules to quasi-in situ tensile properties and internal force balance of cultured smooth muscle cells on a substrate

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Nagayama K, Matsumoto T. Contribution of actin filaments and microtubules to quasi-in situ tensile properties and internal force balance of cultured smooth muscle cells on a substrate. Am J Physiol Cell Physiol 295: C1569–C1578, 2008. First published October 15, 2008; doi:10.1152/ajpcell.00098.2008.—The effects of actin filaments (AFs) and microtubules (MTs) on quasi-in situ tensile properties and intracellular force balance were studied in cultured rat aortic smooth muscle cells (SMCs). A SMC cultured on substrates was held using a pair of micropipettes, gradually detached from the substrate while maintaining in situ cell shape and cytoskeletal integrity, and then stretched up to ~15% and unloaded three times at the rate of 1 μm every 5 s. Cell stiffness was ~20 nN per percent strain in the untreated case and decreased by ~65% and ~30% following AF and MT disruption, respectively. MT augmentation did not affect cell stiffness significantly. The roles of AFs and MTs in resisting cell stretching and shortening were assessed using the area retraction of the cell upon noninvasive detachment from thermoresponsive gelatin-coated dishes. The retraction was ~40% in untreated cells, while in AF-disrupted cells it was <20%. The retraction increased by ~50% and decreased by ~30% following MT disruption and augmentation, respectively, suggesting that MTs resist intercellular tension generated by AFs. Three-dimensional measurements of cell morphology using confocal microscopy revealed that the cell volume remained unchanged following drug treatment. A concomitant increase in cell height and decrease in cell area was observed following AF disruption and MT augmentation. In contrast, MT disruption significantly reduced the cell height. These results indicate that both AFs and MTs play crucial roles in maintaining whole cell mechanical properties of SMCs, and that while AFs act as an internal tension generator, MTs act as a tension reducer, and these contribute to intracellular force balance three dimensionally.

contribution by AFs and MTs in cells would affect the three-dimensional intracellular force balance, and that AF and MT

cellular biophysics; cellular mechanics; hysteresis; cell retraction; cellular prestress

CYTOSKELETAL STRUCTURES such as actin filaments (AFs) and microtubules (MTs) play dominant roles in various cellular events, including cell proliferation (21), migration (18), differentiation (7, 47), and apoptosis (6). They also determine the mechanical properties (50, 56) and shape stability (42, 44) of cells and contribute to physical interactions between cells and extracellular matrices (17). These structures are believed to play crucial roles in the process of intracellular mechanical signal transduction (10). To investigate the signal transduction mechanism in detail from a mechanical point of view, it is very important to determine how much force is transmitted through each of the cytoskeletal structures. For this purpose, quantitative information pertaining to the mechanical properties of the cells under physiological deformation conditions and intracellular force balance is indispensable.

The mechanical properties of cells have been investigated using conventional mechanical testing such as micropipette aspiration (44, 53), magnetic particle twisting (55), and nanoin dentation using atomic force microscopy (20, 45). Several reports have detailed the contribution of AFs and MTs to the mechanical properties of endothelial cells (56), skeletal muscle cells (7), and osteoblasts (51) and have noted that AFs but not MTs affect cell stiffness. However, these studies reported only on the mechanical properties of local regions of cells under small deformation and provided no information on the contribution of the cytoskeleton to whole cell mechanical properties, which are especially important for cells in areas comprising soft biological tissues such as vascular endothelial and smooth muscle cells (SMCs) since arteries are cyclically stretched by ~10% in the circumferential direction due to the pulse pressure. Although the mechanical properties of cells under large deformation have been measured in a single cell tensile test, most of these investigations used cultured cells detached from a substrate by trypsinization (29, 31, 35, 52). Trypsinized cells become round following detachment and lose their shape and cytoskeletal integrity on the substrate, suggesting that serious changes in mechanical properties may occur. It is therefore essential to measure whole cell mechanical properties using cells with maintained in situ cell shape and cytoskeletal integrity on the substrate.

The effects of AFs and MTs on intracellular force balance have been discussed mainly with respect to the two-dimensional traction force exerted by cells on the substrate, which was estimated by measuring cell retraction following cell cutting (40) and measuring deformation of a polyacrylamide gel substrate (49, 57, 58). These studies demonstrated that the traction increased following actomyosin activation, decreased following AF disruption, and increased following MT disruption. These reports indicated that the organization of cytoskeletal structures could be expressed using a simple mechanical model, referred to as the “tensegrity” or “push-pull” model (14, 22), in which AFs are prestretched elastic components generating intracellular traction force with myosin motors and MTs are postulated to function as rigid struts opposing the force generated by AFs. We considered that this type of mechanical contribution by AFs and MTs in cells would affect the three-dimensional intracellular force balance, and that AF and MT

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structural changes would induce three-dimensional morphological changes in cells, which are especially important in terms of functional and phenotypic changes of SMCs (27, 38). However, there have been no reports detailing the effect of cytoskeletons on the three-dimensional intracellular force balance.

In the present study, the tensile properties of cultured SMCs with maintained in situ cell shape on a substrate and cytoskeletal integrity were measured using the quasi-in situ loading/unloading tensile testing method established in our previous study (36). Changes in whole cell stiffness and the apparent viscous energy loss were measured during the test before and following AF disruption, MT disruption, or augmentation of MT polymerization to study the effects of AFs and MTs on the mechanical properties of SMCs. The effects of AF and MT disruption and MT augmentation on intracellular force balance were then investigated. The force balance within the plane of the substrate was evaluated by examining the degree of cell retraction following noninvasive detachment from the substrate coated with the thermoresponsive-gelatin poly N-isopropylacrylamide (PNIPAAm)-grafted gelatin, which dissolves in the medium below 32°C (32, 34). Cells attached on the gelatin layer at 37°C lose their scaffold and are isolated from the substrate by lowering the ambient temperature to below 32°C. Unlike trypsinization, which severs adhesion molecules, this method does not cause unwanted damage to cells that usually induces cell contraction. The three-dimensional force balance was evaluated by examining changes in the height, projected area, and volume of the cells as determined by confocal microscopy. Using these approaches, the contribution of AFs and MTs to whole cell mechanical properties and the internal force balance of SMCs was investigated.

MATERIALS AND METHODS

Preparation of SMCs. Rat aortic SMCs were used as the test model. All animal experiments and treatments were approved by the Committee on Animal Experimentation of Nagoya Institute of Technology and were carried out in accordance with the Guide for Animal Experimentation. SMCs were isolated from aortic tissue using an enzymatic digestion method previously described (35). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (JRH Bioscience) and 1% penicillin-streptomycin (GIBCO) at 37°C in 5% CO₂ and 95% air. After having reached confluence in ordinal plastic culture dishes, cells were harvested by trypsinization and plated on ϕ 60-mm glass-bottom culture dishes (no. 0, Matsumi) coated with poly-D-lysine or PNIPAAm-grafted-gelatin. The cells on poly-D-lysine-coated dishes were cultured for 2–4 days and used for the cell tensile test and confocal microscopy, and those on PNIPAAm-grafted gelatin-coated dishes were used for the cell retraction measurement. It was confirmed that the SMCs cultured on poly-D-lysine-coated dishes for 2–4 days had mature focal adhesions and that the morphology and mechanical properties of these cells did not differ significantly from those cells cultured on a PNIPAAm-gelatin-coated substrate (36). Cells at passages 5–13 were used for all experiments since in a previous study no significant difference was observed in the mechanical properties and morphology of SMCs in these passages (37).

Preparation of PNIPAAm-gelatin-coated dishes. For cell retraction measurements, the noninvasive cell detachment technique was adapted using the PNIPAAm-gelatin-coated dishes as previously described (34, 36). PNIPAAm-grafted gelatin (32) was dissolved in distilled water at 0.09 mg/ml at ~4°C, and PNIPAAm (Sigma-Aldrich) was similarly dissolved in distilled water at 2.2 mg/ml. Both solutions were mixed, sterilized by filtration using a microfilter with a pore size of 0.22 μm (Millipore), and then stored at 4°C until use to avoid condensation. A 100-μl aliquot of the mixed solution was placed onto the glass area (ϕ 14 mm) of the culture dish to coat the surface and was then air-dried on a clean bench for 3–12 h. The PNIPAAm-gelatin-coated dishes were used within 24 h after air-drying.

Quasi-in situ cell tensile test. The cell tensile test was performed using our laboratory-made tester (33, 35). The specimen cell was observed with an inverted microscope (TE2000E, Nikon) and was held with two micropipettes with tip diameters of approximately 5–8 μm. One micropipette was rigid and was moved horizontally with the electric micromanipulator (MMS-77, Shimadzu), while the other micropipette was flexible to measure the force applied to the cells by observing its deflection. The cell stretching process was observed with a cooled digital charge-coupled device (CCD) camera (ORCA-ER, Hamamatsu Photonics) connected to the microscope.

SMCs adhering to the poly-D-lysine-coated dishes were treated with a Ca²⁺-Mg²⁺-free medium (Ca²⁺-Mg²⁺-free Hank’s balanced salt solution; HBSS; Sigma) for 30 min at 37°C, which almost inhibited cell contraction and morphological changes with mechanical stimuli and did not have adverse effects on SMC viability (36). A cell on the dish with a length >100 μm was randomly chosen and held with the micropipettes coated with a urethane resin adhesive (Sista M5250, Henkel) (35, 46) as follows: First, the distance between the pair of micropipettes was set at ~70 μm, and the no-load position of the flexible pipette was recorded. The pipettes were then gently pressed on the surface of the cell body at both end regions (Fig. 1a). Treatment with HBSS did not cause unwanted reduction cell adhesiveness; thus the target cell could be detached from the dish by careful lifting up of the pipettes while maintaining the in situ shape of the cell on the substrate (Fig. 1b). The medium was then replaced with preheated serum-free DMEM, and incubation proceeded for 30–60 min. The cell length was adjusted at no-load by moving the rigid pipette to place the flexible pipette back at the no-load position that had been determined before the test. The cell was then stretched stepwise from the no-load length by moving the rigid pipette 1 μm every 5 s until the strain reached ~15% and was then unloaded at the same rate to the initial position. This cyclic process was performed 3–4 times both before and following drug administration to investigate the effects of AFs and MTs on the mechanical properties of SMCs. The tensile properties of SMCs were examined in serum-free DMEM (tensile test 1), and then either cytochalasin D (2 μg/ml, 1 h), which inhibits AF polymerization; colchicine (0.4 μg/ml, 2 h), which inhibits MT polymerization; or taxol (10 μM, 2 h), which inhibits MT depolymerization, was added to the specimen bath while pipettes were kept at the initial position, and changes in the mechanical properties of the SMCs were measured again (tensile test 2). Measurements were also performed for untreated cells left still for 2 h following tensile test 1. The distance between the two pipettes L was measured, together with the displacement of the deflection pipette x (Fig. 1c). The tension applied to the cell T was calculated by multiplying x with the spring constant of the deflection pipette k, which was measured after each experiment. The strain of the cell ε was calculated as the ratio of cell elongation ε = (L − L₀)/L₀, the mechanical properties of the cells were evaluated using tension-strain (T-ε) curves. A large hysteresis was observed in the first cycle of the loading/unloading process and almost became stable at the second and subsequent cycles, and the curves were somewhat nonlinear, especially in the small strain range (Fig. 2A). To investigate the effects of AFs and MTs on cell stiffness in the small- and large-strain ranges, two types of cell stiffness were calculated: the initial stiffness S₀ as the average slope in the second and subsequent cycles in the range ε ≤ 5%, and the stiffness associated with the large deformation Sₕ in the range ε ≥ 5% where the curves were almost linear. The areas under the curve for loading (Φ₁) and unloading (Φ₄) were measured. The hysteresis, calculated by subtracting Φ₄ from Φ₁,
represents the apparent viscous dissipation during loading and unloading (15). Thus we defined the apparent viscous energy loss $V_{E_{\text{loss}}}$ from the hysteresis area with the area of loading curve $H_{L}$, as given by $V_{E_{\text{loss}}} = \frac{\Phi_L - \Phi_U}{\Phi_L}$. Measurement of cell retraction during detachment. Untreated SMCs and SMCs treated with cytochalasin D (2 μg/ml, 1 h), colchicine (0.4 μg/ml, 2 h), or taxol (10 μM, 2 h) were used to examine cell retraction during detachment from PNIPAAm-gelatin-coated dishes. The specimen dish filled with 8 ml drug-free DMEM was set on the inverted microscope stage. The dish was kept at 37°C using a temperature controller and was covered with a laboratory-made acrylic test chamber to prevent evaporation of the medium and to maintain the humidity in the test section. The SMCs were detached from the dishes by dissolving the PNIPAAm-gelatin with the addition of 2 ml precooled (4°C) DMEM into the dishes. The medium temperature decreased only to 30°C, and cell detachment was complete within ~1 min. Thus, it is believed that the effects of cooling on cell retraction were negligible. Cell images during detachment from the dishes were observed with the CCD camera connected to the microscope and were recorded on a personal computer. Cell detachment was confirmed by gently tapping the microscope stage. After the experiments, cell outlines in these images were manually traced and analyzed using image analysis software (MetaMorph version 6.0, Universal Imaging). Cell length $l$ and area $A$ were measured just before the temperature decrease ($l_a$ and $A_a$) and at the time when the cells were completely detached from the dishes ($l_d$ and $A_d$). The decrease in cell length $\Delta l = (l_a - l_d)/l_a$, and area $\Delta A = (A_a - A_d)/A_a$, was calculated to assess the role of AFs and MTs in resisting cell stretching and shortening (34).

Estimation of three-dimensional cell morphology using confocal microscopy. To observe the three-dimensional morphology of AFs and MTs, SMCs were plated onto poly-d-lysine-coated glass-bottom culture dishes and then treated with cytochalasin D (2 μg/ml, 1 h), colchicine (0.4 μg/ml, 2 h), or taxol (10 μM, 2 h). Cells were then fixed for 10 min using phosphate-buffered saline (PBS, Nissui) containing 3.7% formaldehyde and were then permeabilized for 5 min using PBS containing 0.1% Triton X-100 (ICN Biomedicals). For AF staining, cells were incubated for 30 min at room temperature with Alexa Fluor 546-conjugated phallodin (~200 nM; Molecular Probes). For MT staining, fixed and permeabilized cells were incubated for 2 h at room temperature with monoclonal anti-β-tubulin antibody (1:1,000; Chemicon) in PBS containing 1% bovine serum albumin (Sigma). After cells were washed, they were incubated for 30 min at room temperature with Alexa Fluor 488-conjugated secondary antibody (1:200; Molecular Probes). Optical sections of AFs and MTs were obtained at a 0.25-μm interval using an inverted microscope combined with a confocal laser scanning system (Digital Eclipse C1, Nikon) and an oil immersion objective (×60, 1.40 numerical aperture). Three-dimensional images were reconstructed using image analysis software. The maximum cell height at the nucleus region $H_{\text{max}}$ and cell area $A$ were measured using the projected images for the lateral and vertical directions, respectively (Fig. 3). The cell volume...
was also estimated by measuring the cell area of each confocal image and multiplying these values with the interval of the sections (0.25 μm).

Data analysis. Data are expressed as means ± SD. Statistically significant differences in cell stiffness before and following drug treatments were assessed with paired Student’s t-test. For the cell retraction measurements and confocal microscopy, data were assessed using ANOVA with a correction for multiple comparisons, followed by a Steel-Dwass multiple comparisons of the means between two groups using a statistical analysis program (MEPHAS, http://www.gen-info.osaka-u.ac.jp/MEPHAS/). Differences with \( P < 0.05 \) were considered to be significant for both analyses.

RESULTS

Untreated SMCs appeared as straight, with almost parallel, fiberlike AFs (stress fibers) (Fig. 3A). AFs showed a punctate distribution following cytochalasin D treatment, with an average fluorescent intensity that decreased by \(~70\%\), indicating that although some punctate AFs remained, especially at the cortical region, stress fibers had completely disrupted (Fig. 3B). Treatment with colchicine or taxol did not have a significant effect on AF structures, except that the AFs seemed to appear concentrated at the cell periphery (cf. Fig. 3, A and B). Disruption or augmentation of MTs tends to inhibit colocation of AFs and MTs and causes separation of the two filaments. On the basis of the reconstructed three-dimensional images, the maximum cell height at the nuclear region, projected cell area, and cell volume were measured (Table 1). The maximum cell height doubled following treatment with cytochalasin D (Fig. 3, I and J), decreased by \(~15\%\) following treatment with colchicine (Fig. 3K), and increased by \(~25\%\) following treatment with taxol (Fig. 3L). Although the cell area decreased significantly following treatment with either cytochalasin D or taxol, the cell volume did not differ significantly between the four groups.

Figure 4 shows representative images of AFs and MTs of an untreated SMC that remained stretched by \(~15\%\) following the quasi-in situ tensile test. AFs appeared straight and run from one end of the cell to the other (Fig. 4b). In contrast, MTs appeared concentrated in the region with low AF concentration and did not cover the entire length of the cell (Fig. 4c, arrow). Some of the MTs appeared straight (Fig. 4, d and e, closed arrowheads) while others were curved (Fig. 4, d and e, open arrowheads).

Table 1. Three-dimensional morphology of smooth muscle cells in the four groups

<table>
<thead>
<tr>
<th></th>
<th>Untreated (n = 50)</th>
<th>Cytochalasin D (n = 50)</th>
<th>Colchicine (n = 50)</th>
<th>Taxol (n = 50)</th>
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</thead>
<tbody>
<tr>
<td>Maximum cell height, μm</td>
<td>4.0±0.6</td>
<td>11.4±2.0*</td>
<td>3.3±1.0*</td>
<td>5.5±1.6*</td>
</tr>
<tr>
<td>Projected cell area, μm²</td>
<td>2.870±1.110</td>
<td>1.480±0.490*</td>
<td>2.550±2.920</td>
<td>1.920±1.530*</td>
</tr>
<tr>
<td>Cell volume, μm³</td>
<td>6.430±2.100</td>
<td>9.600±1.450</td>
<td>5.970±1.940</td>
<td>6.390±2.060</td>
</tr>
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</table>

Values are means ± SD. *P < 0.05 vs. untreated.
cell stiffness significantly but led to an increase in the apparent viscous energy loss of 40%.

Figure 6 shows representative images of SMCs before (A–D) and immediately following (E–H) detachment from PNIPAAm-gelatin-coated dishes, and the changes in length Δl and area ΔA are summarized in Fig. 7. Untreated SMCs appeared shortened at both ends to the central region almost along the long axis direction, and they maintained a spindle or rectangular shape even after detachment from the substrate (Fig. 6, A and E). The decreases in length Δl and area ΔA showed the same trends, with Δl/ΔA being ~40% for the untreated cells (Fig. 7). In contrast, cells treated with cytochalasin D shortened only slightly, and Δl/ΔA values were one-half of those of untreated cells (Figs. 6F and 7). After inhibition of MT polymerization using colchicine, the SMCs shortened remarkably, with Δl/ΔA values significantly larger than those of untreated cells (Fig. 6, C and G, and Fig. 7). On the other hand, SMCs subjected to MT depolymerization inhibition using taxol maintained cell shape after detachment and with significantly decreased Δl and ΔA values (Fig. 6, D and H, and Fig. 7).

DISCUSSION

Contributions of AFs and MTs to whole cell mechanical properties. In the present study, SMCs cultured on a substrate were successfully held using glass micropipettes while maintaining in situ cell shape and cytoskeletal integrity (Figs. 1 and 4), and the loading/unloading test for SMCs was performed up to ~15% strain for the first time. A large hysteresis was observed in the first cycle of the test even though the cells were stretched by only ~15%, and the hysteresis decreased in subsequent cycles (Fig. 5, A–D). These results indicate that the mechanical properties of isolated cells just after detachment from the substrate were not stable. In this study, micropipette tips were attached on the upper surface of the cells. Focal adhesion proteins that connect actin stress fibers to the cell membrane exist mainly on the lower surface of cells and facing the substrate. When cells were held on the upper surface, the connections between the pipettes and stress fibers may have been sparse at the beginning. However, as shown in Fig. 4b, stress fibers were running through the cell body, and a substantial amount of AFs were attached to the micropipettes following the loading/unloading cycles. This may indicate that the AFs and pipettes became firmly connected during the stretch test, possibly through the active formation of focal adhesions on the pipette surface and local rearrangement of the cytoskeletons, which may be both active and passive. The relatively large hysteresis observed in the first loading/unloading cycle may have been caused by this type of rearrangement of cytoskeletons to restore the intracellular mechanical environment. It was therefore considered that a mechanical preconditioning process was necessary even if the cells were detached maintaining the in situ cell shape, and the cell stiffness was estimated as the average slope of the loading curve in the stable loops.

The stiffness $S_l$ obtained with the quasi-in situ tensile test (Table 2) was significantly higher than that obtained using trypsinized spherical SMCs (~15 nN/% (29, 35)). Furthermore, on the basis of measurements obtained using confocal microscopy, the cross-sectional area of SMCs on a substrate perpendicular to the stretch direction (72 ± 17 μm², n = 23)
was less than one sixth that of the spherical cells (447 ± 183 μm²; n = 20), which also represents the stiffness normalized by the cross-sectional area of cells and corresponds to the elastic modulus, and was higher in SMCs with in situ cell shape (6–13 kPa) than in trypsinized cells (~3.4 kPa) due to alignment of the actin stress fibers as in situ SMCs (Fig. 3A). Moreover, cell softening with AF disruption was significantly larger in SMCs with an in situ cell shape (Sₗ decreased by ~65%) than in trypsinized cells [Sₗ decreased by ~50% (35)], indicating that the contribution by AFs is essential in maintaining in situ cellular mechanical properties.

The effect of cytoskeleton on cell local stiffness was investigated in several studies by disrupting each cytoskeletal structure. In the surface indentation test using atomic force microscopy, disruption of AFs using cytochalasin D decreased the elastic properties of the cells by ~50%, while MT disruption did not affect their elastic properties (7, 51). Magnetic twisting cytometry revealed that the surface stiffness of endothelial cells decreased to 50% and 20% following AF and MT disruption, respectively (56). These previous studies indicated that MTs had smaller contributions to the local surface stiffness of cells. In our results examining whole cell stiffness, MT disruption did not alter Sₘₙ, as was observed with previous studies, although Sₗ decreased by as much as 30%. This indicates that MT disruption led to a reduction in nonlinearity of whole cell stiffness (the ratio of Sₗ to Sₘₙ). The contribution of MTs to the nonlinearity of cell stiffness could be explained by the following mechanisms: 1) the majority of intracellular MTs were compressed and buckled until the macroscopic strain of cells reached 5% and 2) some of the MTs connected to the cell membrane began to be partly stretched when the cell strain was ~5%. In fact, MT morphology was observed in SMCs being stretched after the quasi-in situ tensile test, and it was found that some of the MTs appeared straightened in a cell stretched by ~15% (Fig. 4, d and e, closed arrowheads). Recent reports have indicated that some capping proteins could

**Table 2. Summary of stiffness and viscous energy loss of smooth muscle cells in the four groups**

<table>
<thead>
<tr>
<th></th>
<th>Untreated (n = 10)</th>
<th>Cytochalasin D (n = 10)</th>
<th>Colchicine (n = 9)</th>
<th>Taxol (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial stiffness Sₘₙ, nN/μm²</td>
<td>13.0±3.4</td>
<td>14.0±3.8</td>
<td>14.3±3.0</td>
<td>13.5±4.4</td>
</tr>
<tr>
<td>Change (test 2 - test 1)</td>
<td>12.7±3.2</td>
<td>5.1±2.6</td>
<td>13.0±2.9</td>
<td>12.8±5.5</td>
</tr>
<tr>
<td>Stiffness in large strain Sₗ, nN/μm²</td>
<td>-2.8±1.4</td>
<td>-57.8±19.8</td>
<td>-8.1±17.9</td>
<td>-9.3±23.2</td>
</tr>
<tr>
<td>Change (test 2 - test 1)</td>
<td>23.3±5.1</td>
<td>24.8±6.8</td>
<td>24.5±5.1</td>
<td>23.5±7.0</td>
</tr>
<tr>
<td>Apparent viscous energy loss during loading/unloading VEₘₙ, %</td>
<td>39.9±11.5</td>
<td>37.2±8.7</td>
<td>38.1±13.7</td>
<td>38.8±8.1</td>
</tr>
<tr>
<td>Change (test 2 - test 1)</td>
<td>40.3±11.3</td>
<td>64.2±10.1</td>
<td>35.8±9.9</td>
<td>55.3±10.5</td>
</tr>
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</table>

Values are means ± SD. Change = (test 2 – test 1)/test 1. *P < 0.05 vs. before treatment.
stabilize and anchor MTs to the cell membrane (41, 59), indicating that some MTs in SMCs may be connected mechan-ically to the cell membrane. Thus there is a possible hypothesis that MTs may have a prominent effect on the whole cell stiffness in the larger strain range, and they may partly be under tension and act as a resistance to stretch when the macroscopic strain of the cell is relatively large. Young’s modulus of single MTs has been reported to be 1.9 GPa (50). Assuming that the inner and outer diameter of MTs is 14 and 25 nm, respectively (50), the stiffness of a single MT is estimated to be ~6 nN/%, which is 25–30% that of the whole cell stiffness obtained in the large strain range (Table 2). Thus, both AFs and MTs play crucial roles in maintaining the whole cell stiffness of SMCs. Future studies are necessary to clarify the dynamic changes of AFs and MTs during cell stretching using GFP-labeled cytoskeletons (1, 16).

Changes in the hysteresis area of the loading/unloading curves were also measured following drug administration in an effort to investigate the effects of AFs and MTs on the apparent viscous energy loss during stretching. It was found that the apparent viscous energy loss increased markedly both with AF disruption and MT augmentation but did not change significantly with MT disruption (Table 2). The cell subjected to AF disruption lost the main component maintaining cell stiffness; thus there was a relative increase in apparent viscous energy loss with the cytoplasmic streaming or reorganization of intracellular structures during the loading/unloading. MT disruption using colchicine also decreased whole cell stiffness, but it did not change the apparent viscous energy loss. In contrast, augmentation of MT polymerization using taxol led to a marked increase in apparent viscous energy loss. It has been reported that the viscosity of microtubule gels increases exponentially with microtubule concentration (54). Thus, the effects of changes in MT density on whole cell viscosity may be larger at high MT concentrations than at low concentrations. With the augmentation of MT polymerization, the mechanical interaction between MT mesh-works and the cytoplasm may have increased. The energy dissipation during cell deformation at low frequencies may not be due to simple fluid viscous dissipation, since at low frequencies, deformation is not entirely passive and does not obey the Stokes-Einstein relationship (4, 13). The energy dissipation may arise due to active reorgan-
zation of cytoskeletal filaments (30). This type of active reorganization of cytoskeletons may have contributed to part of the hysteresis observed in our tensile tests. To make clear the effects of cytoskeletons on the mechanical properties of cells and associated energy consumption during deformation, future investigations will focus on the reorganization of cytoskeletons and the mechanical interaction between cytoskeletons and other intracellular structures.

Contributions of AFs and MTs to intracellular force balance. Cell retraction was successfully measured during noninvasive detachment from a substrate coated with PNIPAAm-gelatin, showing a similar decrease in cell length and area (~40%). This indicates that SMCs on the substrate were generating the intracellular pre-tension mainly in the major axis direction. Changes in cell retraction following drug treatment were also investigated, and it was found that AF disruption decreased cell retraction to less than one-half. In contrast, MT disruption significantly increased cell retraction by ~50%, while inhibition of MT depolymerization decreased cell retraction by ~30%. These results indicate that AFs in combination with myosin generate intercellular tension that compresses other intracellular structures and that MTs can resist this compression. The tensegrity hypothesis (14, 22) is well known as a model that expresses this kind of intracellular force balance where the intracellular pre-tension is carried by AFs, and this pre-tension is hypothesized to be balanced by the combination of interconnected MTs acting as compression struts and the extracellular matrix providing tethering forces. This hypothesis has been supported by two-dimensional studies (49, 57, 58), and the results of our retraction measurements also support this model.

To further investigate the three-dimensional intracellular force balance, changes in maximum cell height at the nucleus region, the cell adherence area, and the cell volume following drug treatment were measured (Table 1). The maximum cell height doubled following AF disruption, and it decreased by ~15% and increased by ~35% following MT disruption and augmentation, respectively. These results indicate that AFs and MTs act as tension generators and resisters, respectively, in the direction of the cell height and that the tensegrity hypothesis could be applied three dimensionally to cells adhering on a substrate (Fig. 8). Another point of interest relating to three-dimensional morphological changes is that the cells maintained their volume following drug treatment and that the increase in cell height following AF disruption and MT augmentation concomitantly induced a decrease in the cell area. This may indicate that the projected cell area is controlled by the balance of intracellular tension in the direction of the cell height and in the direction of the substrate plane while maintaining constant cell volume (Fig. 8, B and D). In contrast, there was no significant change to the projected area of cells subjected to MT disruption even though cell maximum height decreased. This may be due to the cell peripheral region being partly swelled following MT disruption (Fig. 5, G and K, arrowhead), which countered the local volume change caused by the decrease in maximum cell height at the nucleus region. As described above, AFs and MTs play crucial roles in controlling the three-dimensional morphology of SMCs. These cells may experience changes in height and adherence area following changes to the internal force balance when maintaining a constant cell volume.

In this study, we found that MT disruption increased the retraction of SMCs during detachment from a substrate and decreased the maximum height of the cells adhering on the substrate. There are some considerations relating to the nonmechanical effects of MT disruption that should be noted: It has been reported that MT disruption increased the isometric force of cells, an effect attributed to increases in myosin light chain phosphorylation resulting from the release of tubulin monomers (9, 24, 25) or to changes in intracellular Ca\(^{2+}\) (39), which regulate actomyosin contractility. However, cell shortening during detachment from PNIPAAm-gelatin-coated dishes was also measured in the present study in SMCs maximally activated using 10 \(\mu\)M serotonin. The results confirmed that cell shortening increased significantly following MT disruption and decreased following MT augmentation (data not shown). In maximally activated cells, colchicine does not induce further increases in intracellular Ca\(^{2+}\) nor in myosin light chain phosphorylation. Furthermore, a recent study indicated that MTs in living cells can bear enhanced compressive loads due to lateral reinforcement with surrounding elastic filaments and cytoplasm (3). Thus we concluded that MTs act in a mechanical capacity as a resistance to tension borne by other cytoskeletal elements, or for the experiments specific to AF disruption, borne by the AFs.

Although several studies including ours suggest that actin stress fibers are a principal structural component in generating intracellular pre-tension and cell stiffness, other tensed components may be present. The SMCs retracted by 10~20\% after detachment from the substrate even when their stress fibers had
been completely disrupted (Fig. 7). Moreover, although untreated SMCs retracted by ~40% in this study, it has been reported that intact stress fibers experienced 0–20% preextension (8). Recently, the retraction of intact stress fibers in living cells was directly measured after severing using a femtosecond laser (26), and the associated prestrain was estimated to be 19% (28). The difference between the whole cell retraction obtained in this study and that reported for the retraction of intact stress fibers may suggest that mechanisms other than those involving stress fibers may play a role in the intracellular force balance, such as those that may involve surface tension in the cell cortex due to the actin-myosin meshwork. Furthermore, there is the possibility that the nucleus acts as an intracellular compression-bearing organelle (11), and the associated morphology and mechanical properties may affect the intracellular force balance. It has been suggested that intermediate filaments play an important role in maintaining the mechanical stability of cells (2) and in controlling the morphology of cells and nucleus (19). Delineation of the contribution of each intracellular microstructure to the mechanical properties of cells and the intracellular force balance will assist in the establishment of a sound mechanical model of living cells.

In this study, cultured aortic SMCs were used because of their ease of use. It is well known that aortic SMCs change phenotype from contractile and synthetic under culture conditions (5). We previously showed that the elastic property of SMCs decreased to less than one third following a phenotypic change (33, 35); thus the mechanical contribution of each cytoskeleton may change with phenotype differentiation. The contribution of each cytoskeleton to the mechanical properties and internal force balance of contractile SMCs remains to be determined.

**Conclusion.** We investigated the effects of AFs and MTs on the whole cell mechanical properties of SMCs obtained in a quasi-in situ tensile test and associated internal force balance. It was found that both AFs and MTs play crucial roles in maintaining whole cell stiffness of SMCs and have significant effects on cell viscosity. It was also found that while AFs in combination with myosin generate intercellular tension, MTs can resist the tension generated by AFs and contribute to the intracellular force balance three dimensionally, which influences cell retraction, height, and projected area. To make clear the role of AFs and MTs in cell mechanics in more detail, their dynamic changes during the mechanical tests definitely need to be investigated in future studies.

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