Morphological Study of Fibroblasts Treated with Cytochalasin D and Colchicine Using a Confocal Laser Scanning Microscopy

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Abstract: The role of actin filaments and microtubules in 3D cell morphology was investigated using confocal laser scanning microscopy and image analysis based on a region-growing method. Fibroblasts were treated with cytochalasin D or colchicine to disrupt the actin filaments or microtubules, respectively, and the structure and distribution of these cytoskeletal filaments were observed using a confocal laser scanning microscope. From the 3D reconstructed fluorescence images of the cytoskeleton, morphological parameters such as volume, adhesion area, height, and volume ratio of individual cells were determined. The volume ratio was defined as the ratio of the partial volume for every 10% of the height to the total cell volume. The cell volume decreased slightly after the disruption of actin filaments and microtubules, but the change was not significant. The cell adhesion area was significantly decreased after the disruption of actin filaments and microtubules, and was significantly smaller in actin filament-disrupted cells than in microtubule-disrupted cells. Cell height increased significantly after actin filament disruption, whereas it remained almost unchanged after microtubule disruption. Analysis of the volume ratio revealed that the cell shape changed from a cone to a hemisphere after disruption of actin filaments and slightly shifted toward a hemisphere-like shape after microtubule disruption. These results suggest that actin filaments are the major component responsible for the maintenance of global cell shape and that the contribution of microtubules to global cell morphology is much less than that of actin filaments.

Key words: cell, cell volume, mechanics, imaging.

Cells change their shape by alterations of the structure and distribution of the cytoskeleton in response to changes in the local environment. Cytoskeletons are responsible for the mechanical properties of cells and mechanically and functionally interact with one another [1, 2]. In adherent cells, actin filaments are connected to an extracellular matrix via integrins, transmembrane adhesion receptors, at focal adhesions. Forces acting on the extracellular matrix are transmitted to intracellular actin filaments via integrins, and a rearrangement of the cytoskeleton is induced to balance intracellular and external forces [3]. This dynamic rearrangement of cytoskeletons induces changes not only in the shape but also in the function of cells [4]. Cell shape is closely related to the functions and behaviors of cells, including growth, differentiation, motility, and apoptosis. The cytoskeleton mainly consists of actin filaments, microtubules, and intermediate filaments, and each type of cytoskeleton has distinct mechanical properties, dynamics, and biological roles. Therefore, it is very important to understand the contribution of each kind of cytoskeleton to the morphology of cells.

The influence of the extracellular matrix [5–8] and the effects of the degree of polymerization of the cytoskeleton [6, 9–12] on cell shape have been examined using a variety of methods. Many studies have focused on the role of the cytoskeleton in cellular mechanics [5–7, 10–12], and Ingber [13] proposed a cellular mechanical model, the tensegrity model, which focused on the cytoskeletal system. This model can account for some of the mechanical behavior of living cells [7, 14, 15]. The architecture of the tensegrity model consists of stress-supported struts and cable-like structures that have tensile stress, called prestress, in order to maintain structural integrity. The tensegrity model assumes that the prestress that exists even before the application of external forces is generated by actin filaments and is supported by microtubules and components of the extracellular matrices. Although there have been many studies on the roles of components of the extracellular matrices or cytoskeleton in the regulation of cell shape, most have investigated simple parameters such as the projected area and height of cells. However, it is very important to understand the 3D differences in cell shape,
because cytoskeletons are three-dimensionally distributed throughout the cytoplasm. Few studies have examined the 3D shape of single cells [12, 16, 17], and the contribution of the cytoskeleton to the 3D cell shape is still unclear.

In the present study, morphological changes were studied in fibroblasts after disruption of their actin filaments or microtubules, to investigate the contributions of actin filaments and microtubules to the 3D shape of cells. Actin filaments or microtubules were disrupted with cytochalasin D or colchicine, respectively. Morphological parameters were determined with a purpose-built program based on a region-growing method [18] obtained from the confocal fluorescence images of cytoskeletons in adherent fibroblasts.

**MATERIALS AND METHODS**

**Cells.** Fibroblasts were obtained from the patellar tendon of a mature Japanese white rabbit by using an enzymatic digestion method with collagenase, as described previously [19]. Cells were cultured in Dulbecco’s modified Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (PBS, Equitech-Bio, Kerrville, TX, USA), 10 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin (complete medium) in tissue culture flasks. Cells at the 8th to 11th passages were harvested from the flasks using 0.25% trypsin-EDTA solution (Invitrogen) and then cultured on glass coverslips in the complete medium at 37°C for 3 days.

Animal experiments were conducted following the Guideline for Animal Experiments, Graduate School of Engineering Science, Osaka University.

**Treatment of cells with cytochalasin D and colchicine.** To investigate the contributions of actin filaments and microtubules to the 3D shape of the cell, these cytoskeletal filaments were disrupted with cytoskeletal disruptive agents. Fibroblasts that adhered to the coverslip were incubated at 37°C in complete medium containing 10 µg/ml (20 µM) cytochalasin D (Sigma-Aldrich, St. Louis, MO, USA) for 3 h (CD group) or 0.6 µg/ml (1.5 µM) colchicine (Wako Pure Chemical Industries, Osaka, Japan) for 2 h (COL group) to depolymerize actin filaments or microtubules, respectively.

Cytochalasin D has been widely used to disrupt actin filaments in many cell types [6, 14, 20]. The concentrations of cytochalasin D used in these previous studies were 50 pM–2 µM [6], 200 pM–2 µM [20], and 2 µM [14]. In the present study, various concentrations of cytochalasin D were tested to disrupt the actin filaments in the fibroblasts, and lower concentrations were found to be insufficient to cause total disruption (not shown). Therefore, the cells were treated with a relatively high concentration of cytochalasin D (20 µM) to completely disrupt the actin filaments. To disrupt microtubules, colchicine has been used at various concentrations such as 0.1 µM–100 µM [21], 1 µM [15], and 12.5 µM [9]. It was reported that treatment with higher concentrations (>10 µM) of colchicine resulted in the promotion of actin filament formation [21]; therefore, microtubules were disrupted with a relatively low concentration of colchicine (1.5 µM) to avoid enhancement of actin polymerization.

**Confocal laser scanning microscopy.** For the morphological study of cells, actin filaments and microtubules were doubly stained and observed under a confocal laser scanning microscope. After treatment of the cytoskeleton with the disruptive agents mentioned above, fibroblasts on the coverslips were washed with phosphate-buffered saline (PBS) and were fixed with 3.7% formaldehyde in PBS for 20 min. Then they were washed with PBS and permeabilized with PBS containing 0.1% Triton X-100 for 1 min. The fibroblasts were then further washed in PBS and incubated with monoclonal anti-beta-tubulin antibody (Chemicon) in PBS containing 1% bovine serum albumin (BSA, Sigma-Aldrich) for 1 h, followed by rinsing with PBS. The cells were exposed to Alexa Fluor 488-conjugated anti-mouse-IgG antibody (Molecular Probes) in PBS containing 1% BSA for 45 min to stain the microtubules. After the cells were washed with PBS, actin filaments were stained with rhodamine-phalloidin (Molecular Probes) in PBS containing 1% BSA for 20 min and finally the cells were washed with PBS. Any excess solution was removed from the coverslips, and 1 drop of the anti-fade reagent SlowFade Gold (Invitrogen) was applied to each coverslip. The coverslip was mounted upside down on a glass slide and fixed to it with melted paraffin wax.

The fluorescently stained fibroblasts were observed under a confocal laser scanning microscope (FV500, Olympus, Tokyo, Japan) with a 60× oil immersion objective (N.A. = 1.40). Serial cross-sectional images of the cells were obtained at vertical intervals of 0.25 µm. Each cross-sectional image consisted of 1,024 × 768 pixels (each pixel = 0.24 µm) of 24-bit color. Three-dimensional cell images were reconstructed by stacking the serial cross-sectional images into layers. The size of a reconstructed voxel was 0.24 × 0.24 × 0.25 µm³.

To calibrate the scale in the Z-direction, green fluorescent beads (approximately 10 µm in diameter) were observed, and their 3D shapes were reconstructed from serial cross-sectional images. The cross-sectional shape of the beads was almost a circle in the X-Y planes, whereas the bead shape was slightly elongated in the Z-direction, in the X-Z and Y-Z planes. Maximum cross-sectional area and height of the beads were measured to determine the Z-extension ratio of reconstructed images of the beads, where the height was defined as the distance from the bottom to the top of the beads, i.e., the diameter in the Z-direction. The average maximum cross-sectional area was 81.8 ± 11.9 µm² (mean ± SD, n = 22) and the height was 13.4 ± 1.2 µm. We estimated the diameter of each bead from the cross-sectional area assuming a circular cross-sectional shape.
Morphological Study on Fibroblasts

and then the Z-extension ratio of each bead was obtained by dividing the height of each bead by its diameter. The Z-extension ratio of the beads was 1.32 ± 0.05. We assumed that all cells were expanded by 1.32-fold in the Z-direction, and therefore the length in the Z-direction of each voxel was multiplied by 0.76 as a correction factor. The height and cross-sectional area of the beads were determined using the method described in the next section.

Extraction of cell morphology from 3D-reconstructed fluorescence images. The cell morphology was analyzed from the obtained fluorescence images by implementing a region-growing method. The region-growing method groups pixels or voxels into larger regions based on predefined criteria [18]. Briefly, a seed voxel or subregion is selected, and then the region is grown either two-dimensionally or three-dimensionally from the seed voxel by assimilating the adjoining voxels with properties such as grayscale value or color that are similar to those of the seed voxel. This method has been used to extract the surrounding airway for voxels with flag 0 or 1, not only in the Z-direction, but also in the X- and Y-axes.

The method described in this paragraph repeatedly extracted all voxels flagged with 0 or 1. Using these procedures, cell morphology was independently extracted from a series of microscopic fluorescence images and was three-dimensionally reconstructed (Fig. 1D). To confirm the appropriateness of the cell extraction, the extracted cells were visualized three-dimensionally with a data visualization software (Tecplot 360, Tecplot, Bellevue, WA, USA) using the coordinates of voxels constructing the cells.

Morphological study. Morphological parameters, including adhesion area $A$, volume $V$, and height $H$, of the individual cells were determined for 42–51 cells per group. Serial cross-sectional images contained a large amount of noise from, among other sources, limited resolution of the confocal laser scanning microscopy in the Z-direction. Because such noise leads to overestimates of the parameter values, care was taken to exclude noise from the analysis. The images of the cell bottom and the adhesion area of cells were determined as described below. The cross-sectional area $S$ of each cell in each cross-sectional image was obtained by counting the number of pixels within the cell region, and the area ratio $S$ for each cross-sectional image of each cell was calculated by dividing $S$ by $S_{max}$, the maximum cross-sectional area for each cell. Then, the sum of the area ratio $S_{max}$ for each cross-sectional image was calculated by summing the $S$ of all cells in the same cross-sectional image. The image at the bottom of the cells was defined as the cross-sectional image at which $S_{max}$ was maximal, and the cross-sectional area of each cell in that image was defined as the cell adhesion area of each cell. Cell volume was calculated from the number and volume of voxels forming each cell. Cell height was defined as the distance between the bottom and the top of the cell. The value of $H'$ was calculated by dividing $V$ by the adhesion.
was used to evaluate differences among the experimental groups. When significant differences were observed, the Bonferroni correction was used to assess the difference between two groups. A value of \( P < 0.05 \) was considered statistically significant.

**RESULTS**

Actin filaments and microtubules were present throughout the intact cells, except in the nucleus (Fig. 3, A and B). **Fig. 3.** Confocal laser scanning micrographs of intact cells (A–D) and cells treated with cytochalasin D (E–H) or colchicine (I–L). A, E, and I, actin filaments; B, F, and J, microtubules; C, D, G, H, K, and L, merged images of actin filaments and microtubules; A–C, E–G, and I–K, view from the X-Y plane; D, H, and L, view from the X-Z plane after correction of the extension in the Z-direction. Bar, 20 \( \mu \)m.
Morphological Study on Fibroblasts

Fig. 4. Typical 3D views of an intact cell (A), a cell treated with cytochalasin D (B), and a cell treated with colchicine (C).

Thick actin bundles (i.e., stress fibers) existed parallel to the long axis of each cell (Fig. 3A). Actin filaments were observed as straight fibers (Fig. 3A), whereas microtubules had an undulating shape (Fig. 3B).

Actin filaments and microtubules in fibroblasts treated with cytochalasin D (CD group) are shown in Fig. 3, E and F. Stress fibers and long actin filaments in the CD group were almost completely disrupted (Fig. 3E); actin could be seen in the central region of the cell as aggregates and in the peripheral region (Fig. 3E). Microtubules maintained a fibrous structure after treatment with cytochalasin D (Fig. 3F). Cell shape was dramatically changed by actin filament disruption; the cells showed marked contraction and increased height. The shape of the majority of cells became nearly round, with branched protrusions at their periphery.

After treatment with colchicine (COL group), microtubules were almost completely disrupted (Fig. 3J), while actin filaments were still retained in the cells and seemed to be unaffected by colchicine treatment (Fig. 3I). Treatment with colchicine induced retraction of some filopodia, but the procedure did not induce marked changes in cell shape.

Examples of 3D views of reconstructed cells for each experimental group are shown in Fig. 4. The images were created with the voxels determined using a purpose-built program for the region-growing method. The complicated shape and morphological features of the cells in each group were successfully extracted. The morphological parameters of the cells were determined from such 3D-reconstructed cells. The cell volume slightly decreased after the disruptions of actin filaments and microtubules (Fig. 5), but the changes were not significant. The adhesion area decreased significantly after the disruptions of actin filaments and microtubules (Fig. 6), becoming significantly smaller in the CD group than in the COL group. The cell height was significantly larger after the disruption of actin filaments but remained unchanged after microtubule disruption (Fig. 7). The value of $H'$, which is the volume/adhesion area ratio, increased significantly.
DISCUSSION

In the present study, changes in the 3D shape of adherent fibroblasts after treatment with cytochalasin D or colchicine were studied using confocal laser scanning microscopy and image analysis based on the region-growing method. In previous studies on the morphological analysis of cells, the morphological parameters determined included the projected areas of adherent cells and nuclei [6]; the projected cell area and cell volume [16]; the cell volume, surface area, diameter, and height of spherical cells [17]; and the cell height and volume [23]. However, the morphological measurement methods and the parameters measured in these studies are insufficient to evaluate 3D...
Morphological Study on Fibroblasts

changes in the complicated geometry of adherent cells. In order to evaluate 3D changes in cell shape, we extracted the shapes of cells in detail using a modified region-growing method. The volume, adhesion area, and height of cells were successfully determined with this method. The obtained parameters were compared among experimental groups, and the contributions of actin filaments and microtubules to the 3D cell shape were clarified. Our method is thought to be a useful approach for evaluating 3D shape of cells not only in cultured monolayer but also in living tissues to study the physiological changes in functions of cells in response to biochemical and mechanical stimuli.

Cell shape changed dramatically after the disruption of actin filaments with cytochalasin D (Fig. 3, E–H), leading to a significant decrease in cell adhesion area (Fig. 6) and an increase in cell height (Fig. 7). These results agree with those of previous studies in which treatment with cytochalasin D induced a dose-dependent decrease in the projected areas of cells and nuclei [6] and an increase in cell height [23]. In addition to these parameters, in the present study, the volume/adhesion area ratio (Fig. 8) and the volume ratio (Fig. 9) were calculated as morphological parameters. The changes in these parameters suggest that the cytoplasm around the microtubule disruption in the middle and top portions of the cells after actin filament disruption. Actin filaments are linked to the extracellular matrix via integrin receptors and transmit extracellular mechanical forces across the plasma membrane to the inside of cells [24]. They also generate contraction forces in cells [25, 26]. Given that integrins and actin filaments are required for cell adhesion, the strength of cell adhesion to substrates would critically decrease following actin filament disruption, and it is thought that partial detachment of the cell from the substrate occurs. Moreover, the contraction force would decrease markedly, and the influence of cortical tension on cell shape would be relatively increased. This partial detachment and reduction of the contraction force of cells caused 3D changes in the cell shape toward a hemisphere.

Microtubule disruption induced a slight decrease in the adhesion area (Fig. 6) but did not affect cell height (Fig. 7). The decrease in the adhesion area is in agreement with results reported in previous studies [10, 12]. The most likely reason for the decrease in the adhesion area is a disturbance of the mechanical balance in the cell after microtubule disruption. In the tensegrity model, microtubules have been proposed to act as rigid struts that oppose cellular contraction [26]. In this model, microtubules and the extracellular matrix are considered as components that bear the contraction forces of the cell. Microtubule disruption induced an increase in cell traction forces [15], suggesting that part of the contractile stresses that had been supported by the microtubules before microtubule disruption had acted on the substrate.

It is possible that some focal adhesions in the periphery could no longer bear the contraction force without the support of microtubules and that the cell might have detached at these sites. In addition, it has been shown that microtubule disruption strengthens the contractile state of the acto-myosin cytoskeleton [27] and that treatment with high concentrations of colchicine (>10 μM) increases the amount of actin filaments [21]. In the present study, we used a relatively low concentration (1.5 μM) of colchicine and did not observe an influence of colchicine on actin filaments. However, actin filaments might be affected by microtubule disruption and, as a result, the contraction force of the cells might be increased. In the case of actin filament disruption, the adhesion area and height decreased and increased, respectively (Figs. 6 and 7). However, although the adhesion area decreased slightly after microtubule disruption, the cell height remained unchanged. Charras and Horton [23] reported that cell height decreased slightly and insignificantly following the treatment of cells with nocodazole, a microtubule disrupter. Our results on the height of microtubule-disrupted cells are in accordance with theirs. We consider that the difference between the effects of actin filament disruption and those of microtubule disruption on cell shape is attributable to a difference in tension in the actin filaments. The tension in actin filaments of adherent cells is thought to be the force that flattens the cells. Even if microtubule disruption influenced actin filaments, the total tension in the actin filaments after microtubule disruption should not be less than that after actin filament disruption. Although a decrease in the adhesion area induces the release of excess tension in actin filaments and produces a driving force to increase cell height, cell height could be kept constant if the total tension in the actin filaments could negate that force. If microtubule disruption were to enhance the polymerization of actin filaments, and if the tension in actin filaments were to be larger than the force to increase cell height, the cell height should be decreased by microtubule disruption. Thus, the change in cell height in microtubule-disrupted cells may largely depend on the conditions of the treatment with microtubule disruptive agents. In the present study, the treatment condition was selected so as to maximize the depolymerization of microtubules and to minimize the effect on actin filament organization. Owing to the effect of tension in the actin filaments, the cytoplasm around the bottom of the cells shifted not to the top portion but to the middle portion of the cells, and cell height remained unchanged after microtubule disruption. The difference in the 3D cell shape between the cells after actin filament disruption and the cells after microtubule disruption is the most interesting finding in this study, indicating that actin filaments and microtubules have distinct mechanical functions in the maintenance of cell shape. These results suggest that slight variation in the function among cells within a
population may be related to the difference in conditions of actin filaments and microtubules in the cells, because cellular functions are closely related to cell shape.

In the present study, the morphology of cells was analyzed using fluorescence images of actin filaments and microtubules to clearly observe the 3D shape of cells. Strictly speaking, the surface configuration of cells is not completely consistent with the outline of the cytoskeletal structures; however, this does not affect the results because actin filaments and microtubules are present throughout the cells. The cells were cultured on uncoated coverslips to obtain clear images using confocal laser scanning microscopy. In previous studies, it was reported that the substrate affected cell shape [5, 6, 25]. For example, Wang and Ingber [5] found that coating the substrate with fibronectin promoted cell spread in a density-dependent manner and resulted in an increase in the projected cell area. As fibronectin binds to integrin, the adhesion strength of cells should be improved by coating the substrate with fibronectin. In the present study, the adhesion strength of cells to the coverslips was not considered to be high; hence, the cells might possibly detach easily from the coverslips upon disruption of the actin filaments or microtubules. If the 3D shape of cells attached to substrates coated with adhesion molecules such as fibronectin were evaluated after the disruption of the cytoskeleton, other interesting and important phenomena might be observed.

To clarify the contribution of each type of cytoskeleton to the 3D shape of cells in detail, further studies are required. For example, it is considered to be essential that the morphological study of cells after treatment with the agents that stabilize the cytoskeletons or enhance the polymerization of cytoskeletons. Therefore, we are planning to investigate the changes in 3D shape of cells after treatment with the agents such as phalloidin and taxol to enhance the influence of actin filaments and microtubules, respectively.

In conclusion, the complicated 3D shape of adherent cells can be extracted from confocal laser scanning micrographs using the region-growing method. Actin filaments and microtubules make different contributions to cell morphology. Actin filaments are the major component responsible for the maintenance of 3D cell shape, and the contribution of microtubules to global cell morphology is much less than that of actin filaments.

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