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Rearrangement of microtubule network under biochemical and mechanical stimulations

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Abstract

Cells are constantly under the influence of various external forces in their physiological environment. These forces are countered by the viscoelastic properties of the cytoskeleton. To understand the response of the cytoskeleton to biochemical and mechanical stimuli, GFP-tubulin expressing CHO cells were investigated using scanning laser confocal microscopy. Cells treated with nocodazole revealed disruption in the microtubule network within minutes of treatment while keeping the cell shape intact. By contrast, trypsin, a proteolytic agent, altered the shape of CHO cells by breaking the peptide bonds at adhesion sites. CHO cells were also stimulated mechanically by applying an indentation force with an atomic force microscope (AFM) and by shear stress in a parallel plate flow chamber. Mechanical stimulation applied using AFM showed two distinct cytoskeletal responses to the applied force: an immediate response that resulted in the depolymerization and displacement of the microtubules out of the contact zone, and a slower response characterized by tubulin polymerization at the periphery of the indented area. Flow chamber experiments revealed that shear force did not induce formation of new microtubules in CHO cells and that detachment of adherent cells from the substrate occurred independent from the flow direction. Overall, the experimental system described here allows real-time characterization of dynamic changes in cell cytoskeleton in response to the mechano-chemical stimuli and, therefore, provides better understanding of the biophysical and functional properties of cells.

INTRODUCTION

Mechanical and chemical stimulation of cells are essential for the regulation of cell morphology and function. Mechanical stimulation occurs through mechanotransduction process, in which mechanical signals are converted into a cascade of biochemical signaling events [1]. This outside-in signaling has been shown to affect cell proliferation, alignment, differentiation and gene expressions[2–4]. Some of the physiological processes controlled by mechanical forces involve blood pressure regulation, vascular responses to fluid shear

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stress, remodeling of bone, maintenance of muscle, and perception of touch and sound [5–7]. Fundamental understanding of processes involved in mechanotransduction will provide new insight into the structure-function relationship in different cells.

A subcellular system model to investigate the response to mechanochemical stimuli is the microtubule network since microtubules are involved in cellular processes regulated by mechanical forces such as vascular tone [8, 9], cardiac contractility [10, 11] and proliferation of cancer cells [12, 13]. Microtubules have also been shown to play major roles in other processes such as development and maintenance of cell shape and polarity, cell division, cell migration, and cell contraction [14, 15].

In this study, we investigated the dynamic changes in the microtubule network of CHO cells after biochemical or mechanical stimulations. The biochemical stimulants consisted of nocodazole and trypsin, which are known to affect the tubulin polymerization and cell attachment, respectively. Mechanical stimulation was applied on the cells in the form of indentation force exerted by an atomic force microscope (AFM) and shear force in a parallel plate flow chamber. The AFM and the flow chamber were integrated onto a confocal microscope to enable simultaneous imaging to investigate the cell response to the mechanical forces. This experimental platform enabled three dimensional (3-D) imaging of molecular dynamics in subcellular structures in real-time while applying chemical and mechanical stimulations.

MATERIALS AND METHODS

Cell culture and plasmid transfection

CHO cells were maintained in continuous culture in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (Irvine Scientific, Santa Ana, CA), penicillin (100 U/mL; Gibco BRL, Grand Island, NY), streptomycin (100 µg/mL; Gibco BRL), and non-essential amino acids (Gibco BRL). Cells were maintained at 37°C in 5% CO₂. The plasmid pIRESneo-eGFP- α tubulin was generated by P. Wadsworth and purchased from Addgene (plasmid 12298) [16]. CHO cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and selected with 0.6 mg/ml of G418 (Invitrogen).

Live-cell imaging

Imaging of the live CHO cells was performed using an integrated atomic force microscope/scanning laser confocal microscope (Nikon A1R) system. The schematic of the experimental setup is shown in Figure 1A. The confocal microscope images were acquired with a Nikon 60X oil immersion objective (N.A. 1.4) at acquisition rates of 1 to 8 s per frame. Z-stacks were generated from 0.2 – 0.5 µm thick serial sections. Still images were acquired during nocodazole treatment and flow chamber experiments at every 8 seconds and 60 seconds, respectively. The “Perfect Focus” function of the Nikon microscope was applied during imaging to account for drift in the imaging plane. Maximum projection images of trypsinized and indented cells were constructed from Z-stack images using Nikon's NIS-Elements or Volocity (version 6.1.1; Perkin Elmer, USA). In the “trypsin” experiments, Z-stack images were collected every 5 minutes. In the “mechanical indentation” experiments, Z-stack images were captured before and after 1, 5, 10, 20, 30, 40, 50, 60, 70, and 80 minutes of indentation, and 1 min after removal of the indenter. All images were acquired at 25°C. To visualize the fine structures formed by following trypsin treatment, the brightness in Figure 4E was enhanced using the NIS-Elements software's built-in look up tables (LUTs) function. In LUT graphical user interface, maximum range of the pixels used to determine the brightness was reduced from 4095 to 900. As a result, the dark pixels in this

range were ignored, the intensity of the pixels in the selected range was increased and the brightness of the image was enhanced. All images were otherwise contrasted and presented without further manipulation.. Photo bleaching was not a concern in our experiments since the fluorescence intensity did not decrease more than 5% compared to the initial fluorescence intensity in these experiments.

Treatment of CHO cells with biochemical agents

CHO cells were cultured overnight at 37°C in glass bottom dishes from Wilco Wells (model HBSB-3522) prior to the experiment. The dish was infused with complete medium including the biochemical agents to be studied at room temperature. Nocodazole (Calbiochem) and cytochalasin D (Calbiochem) were added to the cell medium to achieve final concentrations of 20 μ M and 5 μ M, respectively. In the trypsinization process, the cell medium was pipetted out and 2 ml of 0.25% trypsin-EDTA solution (Gibco) was added into the dish to dissociate the adherent CHO cells. The cells were imaged prior and immediately after the addition of the chemical agents, and confocal images of the microtubule network were collected accordingly (Figure 1B).

Mechanical stimulation using atomic force microscopy

Atomic force microscopy measurements were carried out using an Asylum Research MFP-3D-BIO atomic force microscope (Santa Barbara, CA, USA) with a closed-loop piezo controller. AFM cantilevers were purchased from Veeco (model MLCT-AUHW; Woodbury, NY, USA), and the V-shaped cantilever with a nominal spring constant of 30 mN/m was used in the experiments after attachment of a glass microbead (50 μ m diameter; Polysciences, Warrington, WA). In a typical force measurement, the AFM cantilever was lowered onto the substrate until the desired loading (indentation) force was reached. AFM cantilever deflection and therefore the indentation force (~20 nN) were maintained for the specified duration. Simultaneous imaging of the indented live CHO cells with the AFM cantilever was performed using the integrated confocal system (Figure 1B).

Analysis of microtubules distribution following cell compression

Microtubule fibers were visualized based on GFP fluorescence in CHO cells transfected with GFP-tubulin. The microtubule fibers were defined as intracellular structures detected with a fluorescence threshold set to the background mean GFP fluorescence intensity value plus three standard derivations ($3 \times \text{SD}$) [17, 18]. The transfected cells were compressed as described above with a glass microbead (~50 μ m diameter) and the three-dimensional (3-D) distribution of microtubule fibers was quantified in serial Z-stacks of confocal images acquired in the same cell before and after indentation. The analysis was performed semi-automatically in Volocity software based on the volume of the microtubule fibers at different time points. The volume measurements were acquired within concentric (3-D) regions of interest (ROI) or rings around the point of indentation (Figure 8A). The rings, each measuring ~5 μ m in width, were placed around a central circular ROI (center). The center ROI measured ~10 μ m in diameter and corresponded to the indentation area ("valley") created by the bead. The center and rings combined covered a total cylinder with a radius of ~20 μ m, essentially accounting for most of the cell being analyzed. Thus, changes in the volume of detected structures within the center and surrounding 3-D rings represent redistribution of the microtubules within and around the indentation area over time after application of force (Figure 8B).

AFM cell elasticity measurements

Force-displacement curves were obtained from 10 CHO cells for each test condition with the bare tip of the AFM cantilever. Hertz model was used to obtain Young's modulus values of

CHO cells by modeling the cell as an isotropic elastic solid and the AFM tip as a rigid cone [19]. According to this model, the force (F)-indentation depth (α) relation is a function of Young's modulus of the cell, E , Poisson's ratio, ν , and half cone angle, θ , as follows: $F = [(E/(1-\nu^2))[2 \tan \theta / \pi][\alpha^2]$ [20]. We fitted the force versus indentation curves acquired experimentally to those estimated by Hertz model to extract Young's modulus of CHO cells before and after application of biochemical agents. Student's T-test was performed on these measurements to determine the significance of change in Young's modulus. In order to avoid the effect of the substrate, the maximum indentation (~500 nm) was kept less than 10% of the total cell thickness in the indentation experiments. In Hertz model calculations; we assumed the half cone angle, θ , to be 36° and Poisson ratio, ν , to be 0.33.

Parallel plate flow chamber measurements

CHO cells, transfected with GFP-tubulin, were cultured overnight at 37°C in tissue culture treated μ -Slide VI flow chambers from Ibidi (AutoMate Scientific, Inc., Berkeley, CA). For the experiments, the flow chamber was transferred to the confocal microscope and a series of images were acquired under static conditions. The effects of shear force on microtubule structure were investigated at shear stress of 0.2 dyn/cm^2 . The cells were imaged using the integrated confocal system until the cells were completely detached from the substrate (Figure 1B). Changes in the cell contact areas with the substrate due to shear flow were quantified at the different time points using the NIS-Elements software's built-in object tracking functions..

RESULTS

Effects of biochemical treatments on the tubulin network in CHO cells

The use of biochemical agents to alter microtubule assembly dynamics in a well-characterized molecular manner can help elucidate the role of microtubules in specifying or regulating cell functions. Certain drugs have been of particular interest recently due to their potential in cancer treatment. These drugs alter the microtubule network drastically during cell mitosis and therefore inhibit the cell proliferation of the cancer cell [21]. Some of these antitumor drugs are colchicine, vinblastine, taxol and nocodazole. In our study, we investigated the dynamics of microtubule destabilizing antitumor drug (nocodazole) and compared its effect to that of a proteolytic agent (trypsin) that leads to loss of adhesion in CHO cells. We investigated the rearrangement of microtubule network by confocal imaging after treatment of CHO cells with nocodazole or trypsin. We also quantified the change of CHO cell elastic modulus in response to these treatments. Nocodazole treatment disrupted the tubulin network within minutes. Nocodazole (methyl [5-(2-thienylcarbonyl)-1Hbenzimidazol-2-yl]) is a synthetic drug which affects the polymerization of microtubule network. The effect of nocodazole on microtubule polymerization is highly concentration-dependent. At high concentrations, nocodazole rapidly disrupts the microtubules by promoting their depolymerization [22–24], while at low concentrations (4–400 nM) nocodazole inhibits microtubule dynamic instability [25]. To visualize the effect of nocodazole on microtubule network, nocodazole ($20 \mu\text{M}$) in DMEM medium was added to CHO cells transfected with a plasmid encoding GFP-tubulin. Figure 2A shows representative confocal image of a CHO cell before application of nocodazole. After the drug was added to the cell medium, nocodazole rapidly disrupted most of the microtubule fibers in 4 minutes (Figure 2B) and completely depolymerized them ~ 8 minutes (Figure 2C). These results are in agreement with previous studies showing that nocodazole alters microtubules but not the actin filaments which are mainly responsible for maintaining cell shape [26, 27]. Consistently with our findings, the general shape of the cell is maintained even after complete depolymerization of the microtubule network. To further demonstrate that the actin filaments are unaffected by the nocodazole treatment, we captured confocal

images of CHO cells before and after nocodazole treatment (20 μ M). The results showed no change in the actin network [Supplementary Figure 1]. This was further supported by the elastic modulus measurements. Cell stiffness or elastic modulus was unaffected by nocodazole treatment (Figure 3). However, treating the cells with an actin destabilizing agent, cytochalasin D, reduced the elastic modulus significantly (Figure 3). We also studied the effects of trypsin on the tubulin network. Trypsin is a serine protease which cleaves proteins at the carboxyl side of the amino acids lysine and arginine. It is commonly used to disassociate adherent cells from the culture dish. In this study, we introduced CHO cells cultured on glass bottom dishes to trypsin-EDTA and recorded the cytoskeletal rearrangements of the cell by confocal microscopy. Figure 4A shows a representative image of microtubule fibers in a CHO cell prior to the trypsin treatment. After 12 minutes, deformation of the cell was evident at the top contact region (Figure 4B), and this region completely separated from the coverslip in 16 minutes (Figure 4C). The contact between the coverslip and the cell was lost on the left side after 18 minutes (Figure 4D). Figure 4E shows the cell at 34 minutes after trypsin treatment where the last peptide bonds anchoring the cell to the substrate in the lower right corner of the image were broken as the cell appeared spherical in shape. The brightness in Figure 4E was enhanced described in the Methods to show the cellular “print” on the coverslip (Figure 4F). This finding demonstrates that tubulin was left on the surface of the glass coverslip during cellular detachment. Moreover, these “prints” provide evidence that microtubules are located at focal adhesion points. Although treatment of CHO cells altered the shape of the cell dramatically, the elastic modulus of the cells was not affected (Figure 3).

Effects of mechanical stimulation on microtubule polymerization in CHO cells

Numerous studies have been devoted to studying the effects of mechanical stimulation on cells and tissues. Compression, tension, bending and fluid shear stress are some of the loading modalities used in these studies [28]. The atomic force microscope (AFM) has been particularly useful for mechanical stimulation studies due to its high sensitivity and capability of application and detecting forces in the piconewton range [29]. AFM has been used to mechanically stimulate a large variety of cells such as hair cells [30], osteoblasts [31], endothelial cells [32], neurons [33], and muscle cells [34]. Recently, confocal microscopy was used in combination with AFM to investigate actin dynamics under stretching conditions [34]. In the current study, we used AFM to apply an indentation force onto CHO cells and we investigated the associated alterations in tubulin network using high-resolution confocal microscopy. We compared the response to mechanical indentation to that of fluid shear stress generated in parallel plate flow chamber. An indentation force was applied onto CHO cells using a spherical glass microbead attached to the AFM cantilever tip to study the effect of mechanical loading on the organization of microtubules. Figure 5A shows a cell prior to load application and the center of the indentation region is marked by a cross. The application of an applied force of ~ 20 nN resulted in an initial initiation of ~ 2 μ m that increased to ~ 4 μ m after 5 minutes (Supplementary Figure 2). Accompanying the deformation of the cell was the immediate displacement of microtubules away from the center of the spherical indenter as indicated by the black arrows in Figure 5B and 5C. Concurrently, microtubule fibers within the main bundle structure and at distal sites (white arrows in Figure 5B) of the indented area were disrupted. After 20 minutes of load application on the cell, new microtubule fibers appeared to form at the periphery along the border of the indentation zone as highlighted by the arrows in Figure 5D. At 40 minutes, additional MT fibers were formed away from the indented area (arrow in Figure 5E). By the 80 minute mark, a ring of dense microtubule fibers is clearly evident at the periphery of the indentation zone (Figure 5F). Three-dimensional rendering and reconstruction of the microtubule dynamics in the indented cell are shown in Supplementary Videos 1 & 2.

To understand the effect of shear force on cell mechanotransduction, CHO cells cultured in a parallel-plate flow chamber were exposed to a shear stress of 0.2 dyn/cm^2 . Figure 6 shows how the two neighboring cells responded to the shear of continuous flow. To highlight the region of cell-substrate interaction, Figure 6 presents the images of the cells that are approximately $1 \mu\text{m}$ above the surface of the glass slide. Figure 6A shows the CHO cells prior to the flow of the medium and the arrow points in the direction of the flow. Figures 6B&C show images at different time points after application of flow. A comparison of Figure 6A and Figure 6B revealed that the lower section of the microtubule network contracted for the cell on the left, while the upper region of the microtubule network contracted for the cell on the right. Figure 6C shows the contracted cells relative to the original position after 75 minutes under flow. Focal adhesion points are clearly visible in Figure 6D which shows that the cell detachment occurs at these locations in a discrete manner. In the same figure, the large residue of the cell on the right hand side shows tearing of the CHO cell due to sudden detachment from the substrate. Overall, CHO cells responded differently to the shear force compared to indentation force where the indentation lead to formation of new tubulin network at the location of indentation over time. The change of the area of the cells under shear flow is given in Figure 7 as a function of time. This figure shows that cell areas reduced by 20% before detaching completely from the substrate. The change in cell area was very slow during the experiment and these areas were reduced by only 20% before completely detaching from the substrate.

DISCUSSION

Our confocal images showed microtubule rearrangement in response to mechanical indentation of CHO cells. This rearrangement involved physical displacement of microtubule fibers within different parts of the indented cells, as well as, the disruption and polymerization of microtubules. To quantify the amount of disruption and polymerization of microtubules following indentation, we determined the volume of microtubule fibers in three-dimensional (3-D) rings or regions of interest (ROI). The ROIs essentially account for the microtubule fibers within concentric cylinders around the point of indentation. Microtubule fibers were defined as GFP-positive intracellular structures with fluorescence intensity above threshold ($3 \times \text{SD}$; see Methods). The change of microtubule fiber volume as a function of time around the indentation zone is shown in Figure 8B. This quantitative analysis confirmed the observation described above; that the microtubules were initially displaced away from the indentation zone due to mechanical compression. As a result, there was 20% decrease in the normalized fiber volume in the “center” zone (C0), immediately beneath the indenter, within 5 minutes of indentation. Fiber displacement out of the center was associated with increased microtubule volume in the inner ring (C1). During the same time frame, the volume of the microtubule fibers decreased in the middle (C2) and outer (C3) rings by 20% and 40%, respectively. This is likely the result of the disruption of the microtubule fibers in parts of the cell corresponding to these rings/cylinders as shown by the white arrows in Figure 5B. In addition to the displacement of the microtubules from C0 to C1, we measured further increase in the volume of microtubule fibers in C1 as time progressed. The change in microtubule volume in C1 was $> 20\%$ after 80 minutes of constant indentation compared to that prior to load application (Figure 8B). It is likely that increase in microtubule volume in C1 after the 50 minute mark was due to microtubule polymerization rather than fiber displacement since the fiber volume in the other regions remains relatively constant.

In this study, we demonstrate the application of scanning confocal microscopy in combination with other techniques to investigate the response of the cellular microtubule network to biochemical and mechanical stimulations. In particular, we show that an integrated AFM/confocal microscopy system offers a powerful tool in cytoskeleton research

as it allows for localized mechanical stimulation of cells with simultaneous high-resolution imaging of cytoskeletal changes in real-time. Our analysis of microtubule dynamics in CHO cells can be extended to investigating other components of the cytoskeletal network such as actin filaments and the immediate filaments to understand the cooperative response of these cellular components to biochemical and/or mechanical stimuli. Moreover, future research will therefore be devoted to cells with specialized functions. Ultimately, the utility of this technique will depend on information derived from large numbers of cell.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Schematic representation of an integrated AFM/confocal microscopy system consisting of a Nikon A1R laser scanning confocal microscope and an Asylum Research MFP-3D-BIO AFM. (B) For the research described in this article, the instrument was operated in conventional confocal microscopy mode, in combination with either an AFM or a parallel plate flow chamber.

Figure 2.

Effects of nocodazole on the organization of microtubule network of CHO cell attached to a glass coverslip. Confocal maximum projection images of a CHO cell transfected with GFP-tubulin were acquired (A) prior to the addition of nocodazole, and (B) 4 minutes and (C) 8 minutes after treatment. Scale bar in the figure is 5 μ m.

Figure 3.

Elastic modulus of CHO cells treated with nocodazole, trypsin or cytochalasin D. p-values were derived from Microsoft Excel's Student's T-test. N=10.

Figure 4.

Effects of trypsin on the organization of microtubule network of CHO cell attached to a glass coverslip. Confocal maximum projection images of a CHO cell transfected with GFP-tubulin were acquired (A) prior to the addition of trypsin-EDTA, and (B) 12 minutes (C) 16 minutes, (D) 18 minutes and (E) 34 minutes after treatment. (F) is a contrast-enhanced image of (E). Scale bar in the figure is $5\mu\text{m}$.

Figure 5.

Changes in microtubule fibers/bundles after indentation of CHO cells. Representative Z-stacks of confocal images (shown as maximum projection) of a CHO cell transfected with GFP-tubulin, before and after indentation at the indicated time points. The images are shown in intensity scale to highlight the changes in the GFP-labeled microtubules over time. Red indicates higher GFP fluorescence intensity and blue represents tubulin structures with lower GFP intensity. Panel (A) shows the cell prior to indentation, and panels (B–E) show the cell after application of an indentation force (~20 nN) at the indicated time points. The cross in the panels marks the center point of indentation. The arrows in B point to regions where depolymerization of microtubule fibers was detected. The arrows in D point to regions of microtubule polymerization after indentation. Scale bar in the figure is 10 μm .

Figure 6. Effects of mechanical shear on the organization of microtubule network of CHO cells. Confocal sectional images of CHO cells transfected with GFP-tubulin were acquired (A) prior to the application of flow (arrows point to direction of flow), and (B) 30 minutes and (C) 75 minutes after the application of flow. (D) Images of surface after the cell detached. All confocal sectional images were acquired approximated $1\ \mu\text{m}$ above the surface of the glass coverslip. The dashed outlines in C correspond to the initial position of the two cells. Scale bar is $5\ \mu\text{m}$.

Figure 7.
Time-dependent change in the area of cell-substrate contact of CHO cells in a parallel plate flow chamber with shear stress of 0.2 dyn/cm^2 .

Figure 8. Kinetics of microtubule fiber distribution in compressed CHO cell. (A) Four regions of interest (i.e., C0, C1, C2, and C3) formed by concentric cylinders are defined in panel A. (B) Time-dependent change in microtubule volume in different regions of a compressed CHO cell.