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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.

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1. 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 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 mechano-chemical 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.

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2. Materials and methods

2.1. 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% CO_2. The plasmid pIRES-neo-eGFP-alpha 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).

2.2. 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 Fig. 1A. The confocal microscope images were acquired with a Nikon 60× oil immersion objective (N.A. 1.4) at acquisition rates of 1–8 s per frame. Z-stacks were generated from 0.2 to 0.5 μm thick serial sections. Still images were acquired during nocodazole treatment and flow chamber experiments at every 8 s and 60 s, 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 min. In the "mechanical indentation" experiments, Z-stack images were captured before and after 1, 5, 10, 20, 30, 40, 50, 60, 70, and 80 min 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 Fig. 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.

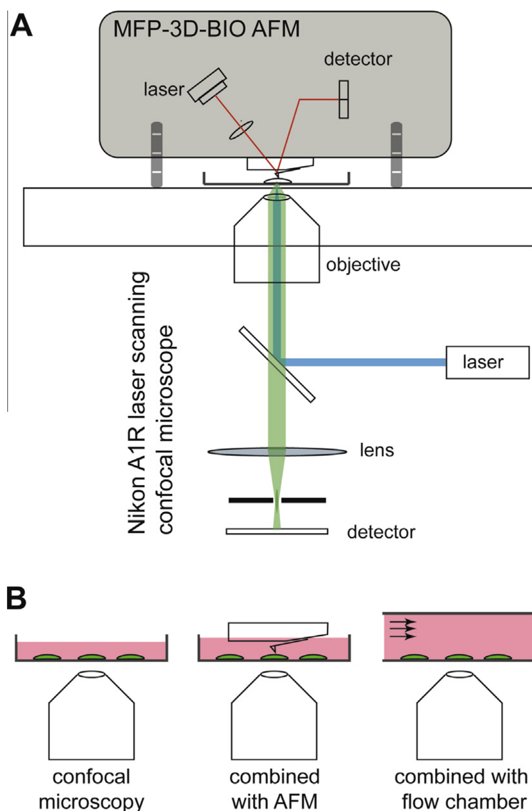

Fig. 1. (A) Schematic representation of an integrated AFM/confocal microscopy system consisting of a Nikon A1R laser scanning confocal microscope and an Asylum Research MFP-3D-BIO AFM. (B) For the research described in this article, the instrument was operated in conventional confocal microscopy mode, in combination with either an AFM or a parallel plate flow chamber.

2.3. 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 μM and 5 μM, 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 (Fig. 1B).

2.4. 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 μm 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 (Fig. 1B).

2.5. 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 × SD) [17,18]. The transfected cells were compressed as described above with a glass microbead (~50 μm 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

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