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Mechanical force-based probing of intracellular proteins from living cells using antibody-immobilized nanoneedles

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ABSTRACT

We developed a method combining atomic force microscopy (AFM) and antibody-immobilized nanoneedles to discriminate living cells by probing intracellular cytoskeletal proteins without the need for cell labeling. The nanoneedles are ultra-thin AFM probes sharpened to 200 nm in diameter. While retracting a nanoneedle inserted into a cell, we measured the mechanical force needed to unbind the antibody–target protein complex. Using this method, the intermediate filament protein, nestin and neurofilament were successfully detected in mouse embryonic carcinoma P19 cells and rat primary hippocampal cells within a minute for a single cell and cell differentiation states could be determined. Additionally, the measured magnitude of the force detecting nestin was indicative of the malignancy of breast cancer cells. This method was shown to affect neither the doubling time of cells nor does it leave extrinsic antibodies within the examined cells, allowing to be used in subsequent analyses in their native state.

1. Introduction

In order to establish the actual state of a given cell type, it is important to probe proteins within the cell without causing the loss of their natural properties. Numerous marker proteins are present both within cells and on the cell surface. Currently, flow cytometry is the most popular technology for analyzing surface proteins of living cells, but it cannot target the considerable number of intracellular proteins that are useful molecular markers of specific cell states. For instance, intermediate filaments are widely known as marker proteins that represent the state of a given cell type (Zehner, 1991). Although fluorescence observation technologies are quite powerful for examining cytosolic proteins, there are currently no label-free methods available for protein detection and typing of living cells.

Recent research has led to the development of nanoneedle technologies for use in the examination of living cells. Ultra-thin needle-shaped materials including fabricated carbon nanotubes have been developed for direct investigation in mammalian cells with extremely minimal cellular damage associated with needle insertion (Chen et al., 2007; Singhal et al., 2011; Yum et al., 2009). The nanoneedle developed by our group is a silicon AFM tip etched to a high-aspect-ratio cylindrical shape with a diameter of 200 nm, and has a high mechanical stiffness that enables repeated penetration of the cell membrane with minimal cell damage. In addition, by employing a perpendicular approach with our AFM nanoneedle, an extremely high insertion efficiency can be achieved (Han et al., 2005; Obataya et al., 2005). Our nanoneedle also has sufficient surface area to harbor a variety of functional molecules (Han et al., 2008; Kihara et al., 2010). AFM has been utilized for analyzing molecular interactions with functionalized AFM tips in a physiological environment (Helenius et al., 2008; Hinterdorfer et al., 1996; Moy et al., 1994). The exterior antigens of target cells can be probed by force measurements using antibody-immobilized AFM tips (Alsteens et al., 2010; Han et al., 2011; Kim et al., 2006; Lee et al., 2007). However, intracellular proteins have not been probed by force measurements using AFM. Here, we report a new technique for mechanically probing intracellular proteins, without the need for cell labeling, using AFM equipped with antibody-immobilized nanoneedles. The technique enables detection of specific intracellular cytoskeletal proteins through the insertion of nanoneedles that have been covalently functionalized with antibodies, and does not leave extrinsic antibodies inside the cell.

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2. Methods

2.1. Cell culture

Mouse P19 embryonic carcinoma cells (Dainippon Sumitomo Pharma Co., Ltd.) were maintained in α-MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM GlutaMAX (Gibco), and gentamicin–amphotericin B (10 μg/mL and 0.25 μg/mL, GA, Cascade Biologics). Mouse NIH3T3 fibroblast cells (Health Science Research Resources Bank) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% FBS, 2 mM GlutaMAX, and GA. The 4T1E, 4T1E/M3, and 4T1E/M8 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in RPMI 1640 medium (Invitrogen) supplemented with 2 mM L-glutamine (Wako Pure Chemical Industries, Ltd.), 1.5 g/L sodium bicarbonate (Invitrogen), 4.5 g/L glucose (Wako Pure Chemical Industries, Ltd.), 10 mM HEPES (Sigma–Aldrich), 1 mM sodium pyruvate (Wako Pure Chemical Industries, Ltd.), and 10% fetal calf serum (Cansera International, Inc.). The cells were treated with PBS containing 0.025% trypsin and 0.01% EDTA and then centrifuged to form a pellet. The cell pellet was dispersed and the cells were seeded onto glass-bottom culture dishes (Asahi Glass Co., Ltd.) and cultured for 1–2 days before use.

2.2. Neural differentiation treatments

For neural differentiation, P19 cells were cultured in a medium containing 1 μM all-trans retinoic acid (Sigma–Aldrich) in bacterial grade culture dishes (AS ONE Corporation) for 4 days. The resulting cell aggregates were seeded onto glass-bottom culture dishes coated with polyethylene-imine and incubated for 5–7 days.

2.3. Preparation of rat primary hippocampal cells

Rat primary hippocampal cells were prepared from Wistar rats (Saitama Experimental Animals Supply Co., Ltd.) on embryonic day 19. Dissociated neurons were seeded onto glass-bottom dishes coated with 0.02% polyethylene-imine (Sigma–Aldrich). Neurons were maintained in 45% Ham's F12, 45% Dulbecco's modified minimum essential medium (Invitrogen), 10% FBS (Invitrogen), supplemented with penicillin–streptomycin (100 units/mL and 100 mg/mL, Invitrogen) and 5 μg/mL insulin (Sigma–Aldrich).

2.4. Preparation of antibody-immobilized nanoneedles

Anti-nestin antibody (MAB353, Millipore) and anti-neurofilament-L antibody (C28E10, Cell Signaling Technology) were used in this study. We prepared nanoneedles reproducibly by fabricating Si AFM cantilever (ATEC-CONT, Nanosensors) pyramidal tips into a cylindrical shape of 200 nm in diameter and 10 μm in length using a focused ion beam as shown Fig. S1 (SMI-9200, Seiko Instruments Inc.). The spring constant of the cantilevers was determined using the thermal noise method (Hutter and Bechhoefer, 1993), and cantilevers with a spring constant of approximately 0.05–0.17 N/m were selected for use. A 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer composed of MPC, γ-methacryloxypropyl trimethoxy silane (MPTS), and p-nitrophenyloxycarbonyl poly-(oxyethylene) methacrylate (MEONP) was used for surface modification of nanoneedles. The mole fraction of the MPC, MPTS, and MEONP in the polymer was 0.69, 0.23, and 0.08, as determined by ^{1}H NMR. Nanoneedles were sequentially cleaned with SPM solution (H_2SO_4:H_2O_2 = 4:1) and APM solution (NH_4OH:H_2O_2:H_2O = 1:1:5) for 15 min at room temperature. Nanoneedles were then dipped into an ethanol solution containing 0.5 wt% MPC polymer, followed by heating at 70 °C for 6 h, after which they were soaked overnight in 10 mM phosphate buffer (pH 8.0) containing 20 nM antibody solution at 4 °C. Before force measurements, nanoneedles were soaked for 30 min at room temperature in PBS containing 1 mM ethanolamine (Sigma–Aldrich) and then washed with PBS.

2.5. Force measurements

Force measurements were carried out using AFM, Nanowizard II BioAFM (JPK Instruments). Antibody-immobilized nanoneedles were inserted into living cells, left to dwell within the cells for 1 s, and then retracted at a tip velocity of 6 μm/s unless otherwise noted. The force exerted on the cantilevers was monitored during both insertion and retraction. For each cell, force measurements were taken 10 times at different positions, and one antibody-immobilized nanoneedle was used for the analysis of 10–20 cells. In some cases no force drop was observed in a force curve, indicating that there was an insertion failure of the nanoneedle. When no force drop was observed, it was not counted among the number of successful insertions. Continuous force measurements using the same nanoneedle against multiple cells caused the observed decrease in the fishing force in P19 cells as cellular debris nonspecifically adsorbed to the nanoneedle surface. Nanoneedles contaminated in this way were regenerated by washing them with PBS containing 0.02% Tween 20.

2.6. Immunostaining

Cells were fixed with 4% formaldehyde (Wako Pure Chemical Industries, Ltd.) for 15 min, washed with PBS, and treated with 0.1% Triton X-100 (Nacalai Tesque, Inc.) in PBS for 2 min. The cells were then washed with PBS three times and treated with 1% BLOCK ACE (Yukijirushi) for 1 h at room temperature. Next, cells were incubated with 0.4% BLOCK ACE solution containing anti-nestin antibody (1:100) for 1 h at room temperature. The cells were washed with PBS and then treated with 0.4% BLOCK ACE solution containing Alexa488-labeled anti-mouse IgG (1:2000, Invitrogen) for 1 h. Finally, cells were washed with PBS and then observed on a fluorescence microscope equipped with a cooled CCD camera system (DP 30/IX71, Olympus Corporation).

2.7. Preparation of nestin-epitope-containing polypeptide and blocking antibody

We synthesized a recombinant polypeptide (NES256) containing an epitope of the tail domain of mouse nestin spanning amino acids 612–867 (Marvin, 1995). The cDNA was prepared from P19 cell total RNA using reverse transcription PCR with the following primers: forward = 5′-GGGGGGCATATGCAGAACCATGAGACCCCAGGA-3′, and reverse = 5′-GGGGGGGGATCCGATTCGAGGGAGACCTGGCT-3′. The cDNA was purified using a Wizard SV gel and PCR Clean-Up System (Promega) and then digested with *Bam*HI and *Nde*I. The digested cDNA was cloned into the pET-15b vector (Merck) and then transformed into *E. coli* strain BL21(DE3)pLysS (Invitrogen). The transformant was cultured in LB broth supplemented with 50 μg/mL ampicillin at 37 °C under induction of isopropyl-1-thio-β-D-galactopyranoside. The transformants were resuspended in PBS containing protease inhibitors (Complete Protease Inhibitor Cocktail, Roche) and then disrupted using ultrasonication. The lysate was centrifuged at 30,000 × *g* for 20 min and then the supernatant was collected. The NES256 polypeptide was purified using a His GraviTrap column (GE Healthcare) according to the manufacturer's instructions. Anti-nestin antibody-immobilized nanoneedles (Anti-NES nanoneedles) were immersed in a 5 μM solution of NES256 and incubated at 4 °C overnight, after which the nanoneedles were washed with PBS.