Biochemical and Biophysical Research Communications 451 (2014) 107–111

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Measurement of cell adhesion force by vertical forcible detachment using an arrowhead nanoneedle and atomic force microscopy

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ARTICLE INFO

Article history:
Received 3 July 2014
Available online 24 July 2014

Keywords:
Cell adhesion force
AFM
Arrowhead nanoneedle
Nanofilm

ABSTRACT

The properties of substrates and extracellular matrices (ECM) are important factors governing the functions and fates of mammalian adherent cells. For example, substrate stiffness often affects cell differentiation. At focal adhesions, clustered–integrin bindings link cells mechanically to the ECM. In order to quantitate the affinity between cell and substrate, the cell adhesion force must be measured for single cells. In this study, forcible detachment of a single cell in the vertical direction using AFM was carried out, allowing breakage of the integrin–substrate bindings. An AFM tip was fabricated into an arrowhead shape to detach the cell from the substrate. Peak force observed in the recorded force curve during probe retraction was defined as the adhesion force, and was analyzed for various types of cells. Some of the cell types adhered so strongly that they could not be picked up because of plasma membrane breakage by the arrowhead probe. To address this problem, a technique to reinforce the cellular membrane with layer-by-layer nanofilms composed of fibronectin and gelatin helped to improve insertion efficiency and to prevent cell membrane rupture during the detachment process, allowing successful detachment of the cells. This method for detaching cells, involving cellular membrane reinforcement, may be beneficial for evaluating true cell adhesion forces in various cell types.

1. Introduction

Cell substrate adhesion is achieved by focal complexes, focal adhesions and fibrillar adhesions, and linkages to the extracellular matrix (ECM) are mediated by integrins and membrane-associated molecules [1,2]. These formations affect cell function, including signaling mechanisms that direct cellular proliferation and differentiation [3–5]. The fate of cellular differentiation can be controlled by the adhesiveness of the substrates [6].

In order to investigate the state of cell adhesion, several methods to detach cells have been reported using centrifugal force [7] or shear stress [8]. However, the results of these methods remain semi-quantitative, since the methods of the force measurement were indirect. Approaches to measure the force more directly have been also reported. One is to detach cells forcibly by cantilever in a horizontal direction [9,10]. Although the force could be directly recorded from the cantilever deflection, this method was not applicable for the strongly adhesive flat cells due to the failure in cell detachments with the horizontal force. As a method to detach cells without failure, Nanofork was reported as a powerful tool to lift cells up completely at once [11]. But it needs a line micropatterned substrate for cell culture and there the cell–substrate adhesion force seems to be Weder et al. detached cells in the vertical direction using a fibronectin-coated flat AFM cantilever in order to obtain a contact area similar to the cell–substrate contact area [12,13]. However, this method applies mechanical stress to the target cells for 30 min in order to anchor fibronectin and integrins before detachment. A hollow cantilever in fluidic force microscopy has also been utilized to quantify the cell adhesion force [14], but the lowest efficiency of cell–substrate detachment was 50% in the measurement (personal communication). Because undetached cells cannot be used to measure cell adhesion force, it is difficult to estimate the true average cell adhesion force.

In this paper, we introduce a novel method using an arrowhead nanoneedle controlled by an AFM to measure cell adhesion force.

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http://dx.doi.org/10.1016/j.bbrc.2014.07.078
0006-291X/

The arrowhead nanoneedle could hook the cortex structure under the cellular membrane, and succeeded in detaching the cells forcibly in the vertical direction with AFM. By forming a nanofilm composed of ECM molecules on the cell surface, cellular membranes were protected from breakage by the arrowhead nanoneedle. This technique allowed the successful detachment of cells and measurement of the adhesion force for various adherent cell lines, including strongly adhesive cells.

2. Material and methods

2.1. Cell culture

Mouse embryonic carcinoma P19 cells (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) were maintained in MEM with alpha modification (α-MEM; Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Carlsbad, CA), 2 mM GlutaMAX (Gibco, Life Technologies) and 0.25 μg/mL gentamicin and amphotericin B (GA; Cascade Biologics, Portland, OR). One day prior to the experiment, cells were treated with 0.25% trypsin and 0.01% EDTA, and were then centrifuged to form a pellet. Mouse mesenchymal C3H10T1/2 cells (JCRB0003; Health Science Research Resources Bank Tokyo, Japan), mouse fibroblast NIH3T3 cells (IFO50019; Health Science Research Resources Bank) and normal rat kidney cells NRK (RCB0043; Institute of Physical and Chemical Research Saitama, Japan) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma–Aldrich) supplemented with 10% FBS, 2 mM GlutaMAX and 10 μg/mL GA. The normal gastric mucosa cell line, RGM-1, which was established by Shimokawa et al. [15], was maintained in DMEM/F-12 (Gibco, Life Technologies) supplemented with 10% FBS (Gibco, Life Technologies), 2 mM GlutaMAX and 1% penicillin–streptomycin (Gibco, Life Technologies).

2.2. Cell preparation for measurement of cell adhesion force

Cells were precultured for 1–2 days and were treated with 0.25% trypsin-1 mM EDTA, followed by pelleting by centrifugation. The pellet was resuspended in culture medium with FBS, and 5×10^4 cells were seeded onto 35-mm polystyrene culture dishes (Iwaki, Tokyo, Japan). Cells were then incubated at 37 °C for 4 or 24 h in a CO_2-controlled incubator prior to measurement of cell adhesion force. Cell detachment experiments were performed in culture medium supplemented with 10 mM 4-(2-hydroxaethyl)-1-piperazineethanesulfonic acid (HEPES) at 37 °C in a temperature-controlled chamber.

2.3. Preparation of arrowhead nanoneedles

Arrowhead nanoneedles were fabricated from the pyramidal tips of standard AFM silicon cantilevers, (OMCL-AC200TN-C3; Olympus, Tokyo, Japan) etched into an arrowhead-shaped end using an FIB (SMI500; Hitachi High-Tech Science, Tokyo, Japan). The width and height of the arrowhead were 2.0 and 1.6 μm, respectively, and the needle was approximately 8–10 μm long and 300 nm in diameter. Spring constants, 1.2 ± 0.3 N/m, were determined using the thermal fluctuation method prior to each experiment. Arrowhead nanoneedles were cleaned with an oxygen plasma asher (JPA300; J-SCIENCE LAB, Tokyo, Japan) at 100 W for 10 min.

2.4. Measurement of single cell adhesion force

Insertion of the arrowhead nanoneedles into the single adherent cells was carried out using AFM (Nanowizard II BioAFM; JPK Instruments, Berlin, Germany) equipped with a Cellhesion module, which enables the probe to move vertically up to 100 μm. All cell manipulations were carried out in a temperature-controlled chamber at 37 °C. Both approach and retraction of the arrowhead nanoneedle were performed at a velocity of 5 μm/s. The force exerted on the cantilever during the insertion and retraction process was monitored using force spectroscopy mode.

2.5. Formation of nanofilms on cell surfaces

Nanofilms composed of fibronectin (fibronectin from bovine plasma; Wako, Osaka, Japan) and gelatin (Wako) (FN-G nanofilms) were formed on cell surfaces using a layer by layer technique, as described previously [16]. Cells were plated in culture dishes at a density of 5×10^4 cells per 35-mm dish, 1 day prior to cell surface laminating. After washing with PBS, cultured cells were incubated in PBS containing 0.04 mg/mL fibronectin for 1 min at room temperature. Cells were then washed with PBS, and were incubated in PBS containing 0.04 mg/mL gelatin for 1 min at room temperature. These consecutive treatments with fibronectin and gelatin were conducted 9 times to form FN-G nanofilms (FN-G9) on the cell surface.

2.6. Actin and fibronectin staining and confocal laser scanning microscopy

For actin staining, all of the following procedures were performed at room temperature. Cells were first washed with PBS, and were then fixed with 4% paraformaldehyde (Wako) for 15 min, followed by three washes with PBS. After permeabilization with 0.1% Triton X-100 (Nacalai Tesque, Kyoto, Japan) for 2 min, cells were washed with blocking solution containing 1% Block Ace (Snow Brand, Tokyo, Japan). For actin staining, cells were incubated with rhodamine–phalloidin (Cytoskeleton, Inc., Denver, CO) for 1 h. For fibronectin staining, cells were incubated with anti-fibronectin antibody (Cosmo Bio Co., Ltd., Tokyo, Japan) for 1 h, and were labeled with secondary antibody (Alexa488-labeled anti-rabbit IgG; Molecular Probes, Life Technologies) for 1 h. Finally, cells were washed with PBS and inspected using a confocal laser-scanning microscope (CLSM; FV-300/IX71; Olympus, Tokyo, Japan) equipped with a CCD camera (DP70-IFAD). Stacked CLSM images at 0.5 μm intervals were acquired, starting from 1–2 μm beneath the bottom of the cells to a vertical height of around 15–20 μm.

3. Results and discussion

3.1. Measurement of cell adhesion forces

In order to measure the cell adhesion force, an arrowhead nanoneedle was inserted into a target single cell and was pulled by the AFM. When the arrowhead nanoneedle was inserted into the cell, a steep force drop appeared on the force–distance curve. The CLSM image confirmed that the arrowhead nanoneedle labeled with green fluorescent protein (GFP) was successfully inserted into NIH3T3 cells expressing red fluorescent protein (Keima-Red) localized on the cell membrane [17]. The arrowhead tip of 2 μm in width could hook cell membrane and lift the cell up (Fig. 1A), otherwise arrowhead tip ruptured cell membrane and was evacuated from the cell. In both cases, a steep peak pulling force appeared on the force–distance curve during needle withdrawal (Fig. 1B and C). The success of cell fishing was confirmed by a phase-contrast view of the needle with or without the cells following this process. We defined the peak forces as the cell adhesion

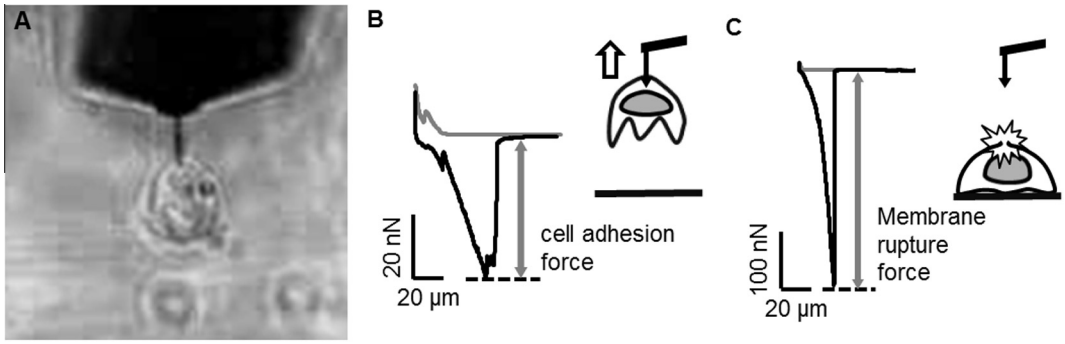

Fig. 1. Definition of adhesion force and membrane rupture force. (A) Lateral image of cell detachment using an arrowhead nanoneedle and AFM. (B) Cell adhesion force. (C) Cell membrane rupture force.

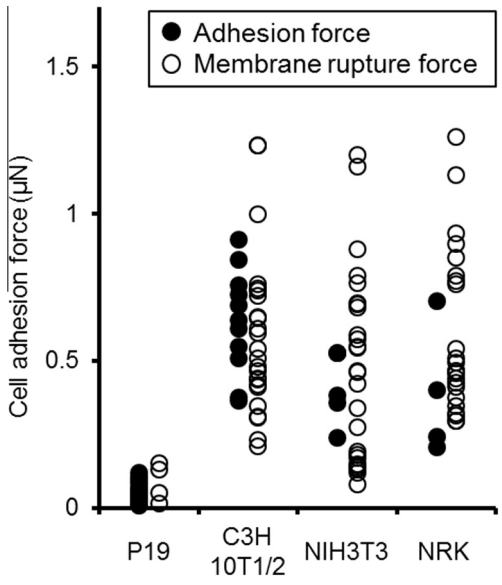

Fig. 2. Plots of adhesion force and membrane rupture force.

force and the membrane rupture force in the case of successful cell fishing and failure, respectively.

Fig. 2 shows the plots of adhesion and membrane rupture forces for various cell lines. The cell adhesion force for P19, NRK, NIH3T3 and C3H10T1/2 was 37, 390, 470 and 630 nN, respectively (Table 1). While only 10% of P19 cells did not detach, more than 70% of the other three types of cells failed to detach. Because the cells that did not detach showed apparently large adhesion forces that could not be measured, it is essential to detach all of the target cells in order to obtain a true average value for the adhesion forces for each specific cell line. Therefore, the average cell adhesion force for C3H10T1/2, NIH3T3 and NRK cells in Table 1 does not show the true average value due to undetached cells with high adhesion forces.

3.2. Strengthening the cell membrane surface using nanofilm

The cytoskeleton is composed of a series of intracellular proteins that maintain cellular shape. We previously showed that the probability of nanoneedle penetration through the plasma membrane greatly depends on the integrity of the meshwork consisting of actin filaments beneath the membrane [18]. Fig. S1a shows the actin filaments of P19, NIH3T3 and C3H10T1/2 cells. Based on these results, it is likely that the cell adhesion force is correlated with the integrity of the actin meshwork in the cell cortex. In C3H10T1/2 cells, which contain abundant actin filaments in the cell cortex, it was possible to detach the cells with adhesion forces of over 800 nN without cell membrane rupture. This suggests that high-density actin filament in cell cortex prevented cell membrane rupture using the arrowhead nanoneedle.

In a previous study, we formed nanofilms composed of ECM molecules on cell surfaces, and found that the formation of nanofilms improved the insertion efficiency of the nanoneedle into fibroblasts and neural cells [19]. This suggests that the nanofilm directly reinforces the cell membrane surface. To prevent cell membrane rupture by the arrowhead nanoneedle during cell fishing, we employed this nanofilm technique by alternately layering cationic fibronectin and anionic gelatin (FN-G9) (Fig. 3A). The CLSM image of the FN-G9 nanofilm laminated NIH3T3 cells after immunostaining with anti-fibronectin antibody showed that the nanofilm is on the cell membrane surface, not under the cell (Fig. S1B). The distribution of cell adhesion forces for NIH3T3 and RGM-1 before and after nanofilm formation is shown in Fig. 3B. The lower range of adhesion forces did not shift higher, indicating that the nanofilm did not form beneath the cells and did not have any effect on cell–substrate adhesion. Furthermore, P19 cells which can be detached from substrate entirely were investigated in order to estimate influence of FN-G nanofilm upon cell adhesion. No obvious change was observed in the adhesion force distribution after nanofilm laminating (Fig. S2).

After FN-G9 lamination, the detachment efficiency of NIH3T3 and RGM-1 cells improved markedly from 26% and 20% to 87% and 53%, respectively (Table 2), showing that strongly adherent

Table 1
Cell detachment efficiency and average force of cultivated cells.

Cell line	Detachment efficiency (%)	Adhesion force (nN)	Membrane rupture force (nN)
P19	89	37 ± 29	87 ± 64
C3H10T1/2	26	630 ± 190	580 ± 270
NIH3T3	22	470 ± 140	490 ± 330
NRK	14	390 ± 230	580 ± 240

These results were obtained at 4 h after cell seeding.

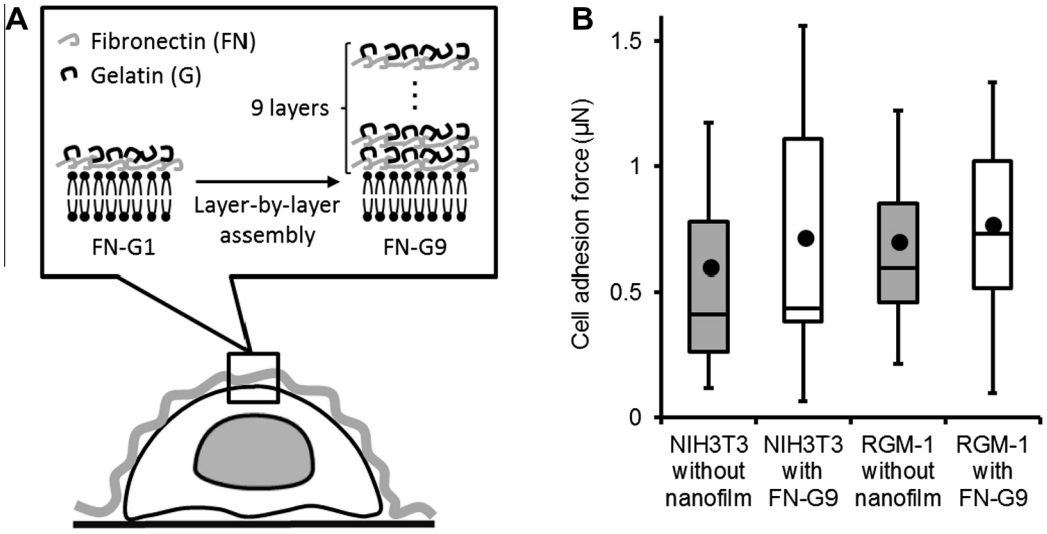

Fig. 3. Strengthening of cell membrane surface by nanofilm. (A) Fabrication process of nanofilms onto cell membrane surface. Nanofilms were composed of fibronectin and gelatin. (B) Box plots of adhesion force of laminated cells. Dots show average cell adhesion force, while dots in boxes show the average adhesion force for each cell line.

Table 2
Cell detachment efficiency and average force of nanofilm-laminated cells.

Cell line	Detachment efficiency (%)	Adhesion force (nN)	Membrane rupture force (nN)
NIH3T3 FN-G0	26	540 ± 390	200 ± 130
NIH3T3 FN-G9	87	670 ± 490	1300 ± 730
RGM-1 FN-G0	20	650 ± 350	500 ± 310
RGM-1 FN-G9	53	720 ± 360	840 ± 360

These results were measured at 24 h after cell seeding.

cells could be detached after nanofilm formation. The average cell adhesion force of NIH3T3 and RGM-1 cells also increased, from 540 nN and 650 nN to 670 nN and 720 nN, respectively. The adhesion force of NIH3T3 cells shown in Tables 1 and 2 was respectively measured at 4 h and 24 h after cell seeding. This cell line with strong adhesion force reached a stable adhesion level at 4 h. The detachment efficiency of NIH3T3 at 24 h after seeding showed almost same when compared with 4 h. Because nearly 90% of NIH3T3 cells were detached after FN-G9 treatment, we believe that an average adhesion force of 670 nN is the true average value for NIH3T3 cells. The detachment efficiency also improved, to more than 30% in the case of RGM-1; however, further improvement is necessary in order to measure the true average value. Similarly, the average adhesion force of 473 nN in HeLa cells measured by Potthoff et al. does not appear to reflect the actual the adhesion force, as they also could not pick up all the cells from the substrate. This method of reinforcing the cell cortex may contribute to obtaining true adhesion force values, even with other methodologies, although optimization of nanofilm laminating conditions for each cell line is necessary.

4. Conclusions

We developed a method to detect cell adhesion force using an arrowhead nanoneedle. The adhesion force could be measured when the target cells were picked up using this arrowhead nanoneedle. As it is essential to prevent cell membrane rupture during the process, a nanofilm consisting of fibronectin and gelatin was used to reinforce the cell membrane surface. After nanofilm lamination, cell detachment efficiency was markedly improved, allowing about 90% of NIH3T3 cells to be detached and their adhesion force to be measured.

Acknowledgments

This research was supported by the Japan Society for the Promotion of Science (JSPS) through the "Funding Program for Next-Generation World-Leading Researchers (NEXT Program)", initiated by the Council for Science and Technology Policy (CSTP).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.078.

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