Journal of Biomechanics 46 (2013) 1081–1087

Contents lists available at SciVerse ScienceDirect

Journal of Biomechanics

journal homepage: www.elsevier.com/locate/jbiomech
www.JBiomech.com

The effects of confluency on cell mechanical properties

Yu.M. Efremov*, A.A. Dokrunova, D.V. Bagrov, K.S. Kudryashova, O.S. Sokolova, K.V. Shaitan

M.V. Lomonosov Moscow State University, Faculty of Biology, Department of Bioengineering, Leninskie Gory, 1/73, 111991 Moscow, Russia

ARTICLE INFO

Article history:
Accepted 21 January 2013

Keywords:
AFM
Vero cells
Viscoelasticity
Confluency
Actin cytoskeleton

ABSTRACT

Mechanical properties of cells depend on various external and internal factors, like substrate stiffness and surface modifications, cell ageing and disease state. Some other currently unknown factors may exist. In this study we used force spectroscopy by AFM, confocal microscopy and flow cytometry to investigate the difference between single non-confluent and confluent (in monolayer) Vero cells. In all cases the stiffness values were fitted by log-normal rather than normal distribution. Log-normal distribution was also found for an amount of cortical actin in cells by flow cytometry. Cells in the monolayer were characterized by a significantly lower (1.4–1.7 times) Young's modulus and amount of cortical actin than in either of the single non-confluent cells or cells migrating in the experimental wound. Young's modulus as a function of indentation speed followed a weak power law for all the studied cell states, while the value of the exponent was higher for cells growing in monolayer. These results show that intercellular contacts and cell motile state significantly influence the cell mechanical properties.

1. Introduction

Atomic force microscopy (AFM) is widely used in biological studies for visualization of biomolecules (Engel and Muller, 2000; Graham et al., 2010) and cells (Costa, 2006; Efremov et al., 2011; Rotsch and Radmacher, 2000) and for the assessment of their mechanical characteristics (Kuznetsova et al., 2007; Rico et al., 2008; Takai et al., 2005). Force spectroscopy of cells by AFM, along with other techniques like the optical stretcher (Guck et al., 2005), micropipette aspiration (Ward et al., 1991), magnetic twisting cytometry (Alenghat et al., 2000) and laser tracking microrheology (Hoffman et al., 2006), is an important source of information about cell mechanical properties. The data about cell mechanical properties combined with confocal microscopy data confirm that the cytoskeleton, mainly the actin network, contributes significantly to cell stiffness (Guck et al., 2005; Mackay et al., 2012; Rotsch and Radmacher, 2000). It has been shown, that changes in cell stiffness are closely related to cell type and various external and internal conditions, like substrate stiffness (Franze et al., 2009; Mackay et al., 2012; Solon et al., 2007) and surface modifications (Takai et al., 2005), cell ageing (Starodubtseva, 2011) and disease state (Lekka et al., 2012). However, the elasticity modulus values measured by AFM additionally depend on several factors, including force loading rate, indentation depth, probe geometry, models for force curve approximation, properties of the substrates used for cell growth and others (Lekka et al., 2012; Mahaffy et al., 2000; Rico et al., 2008). Their influence on the elastic properties of cells must be considered for a proper evaluation and for further comparison of data obtained in different experiments.

In vivo cells stay in close connection with each other in the tissues and continuously exchange signals with neighboring cells (Lodish et al., 2000). Cells, cultivated on a flat substrate *in vitro*, are deprived of some of those signals. Single non-confluent cells are deprived more than cells in a monolayer, where signaling information can be shared by neighboring cells with intercellular contacts. These molecular or mechanical signals may lead to different properties of non-motile cells in monolayer (Yeung et al., 2005) and single non-confluent cells. On the other hand, transition from a non-motile to a motile state is accompanied by polarization of cells. Various signaling and structural proteins operating together lead to the reorganization of the actin cytoskeleton (Kole et al., 2005; Ridley et al., 2003).

We used the African green monkey *Cercopitecus aethiops* kidney cell line (Vero) as a model system to determine the effects of confluency on the mechanical properties of cells. Vero cells are widely used in various areas of biology and biotechnology. They are used as substrates for viral vaccine manufacture (Barrett et al., 2009; Ehrlich et al., 2012), for virus detection (Macfarlane and Sommerville, 1969), for detection of verotoxin (Maniar et al., 1990), as substrates

* Correspondence to: M.V. Lomonosov Moscow State University, Faculty of Biology, Department of Bioengineering, Leninskie Gory, 1/73, 111991 Moscow, Russia. Tel./fax: +79459395738.
E-mail addresses: yu.efremov@gmail.com, efremov@mail.bio.msu.ru (Yu.M. Efremov), aitsanochka-79@yandex.ru (A.A. Dokrunova), dbagrov@gmail.com (D.V. Bagrov), rekamoskva@mail.ru (K.S. Kudryashova), sokolova184@gmail.com (O.S. Sokolova), shaitan@moldyn.org (K.V. Shaitan).

0021-9290/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jbiomech.2013.01.022

for human early round spermatids differentiation (Tanaka et al., 2009) and for the improvement of mouse (Lai et al., 1992) and human early embryo development (Menezo et al., 1990). Vero are non-polarized epithelial cells, which have a typical fibroblastoid shape and form a well-defined flat monolayer. The mechanism by which Vero cells became immortal is still unknown. During tissue-culture passage (at the high passage levels, $p > 200$), Vero cells can develop the capacity to form tumors (Manohar et al., 2008).

Here, we determined the mechanical properties of single non-confluent Vero cells by AFM and compared it with the properties of fully confluent cells (in monolayer). The single cells were prepared either by seeding or by wound healing assay, when cells migrated into the scratch wound from monolayer (Valster et al., 2005). Confocal microscopy and flow cytometry were used to assess the changes in the actin cytoskeleton organization and actin amount.

2. Materials and methods

2.1. Cell culture and confocal microscopy

Vero cells were grown in DMEM medium (PanEco, Russia) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA) at 37 °C in a humidified 5% CO_2 atmosphere in an incubator (Sanyo, Japan). For AFM imaging and force spectroscopy, the cells were harvested from the subculture, seeded on sterile 35 mm Petri dishes and placed into the incubator. For the experiments with single non-confluent cells they were grown for 1–2 days, for the experiments with a monolayer—for 3–4 days. The monolayer wounding was used to examine cell elasticity during migration inside the wound (Valster et al., 2005). Two (or more) parallel scratch wounds of approximately 400 μm width were made with a blue pipette tip (Greiner Bio-one, Germany), the growth medium was replaced and cells were incubated for several hours before AFM measurements.

For the confocal fluorescence imaging, cells were seeded on sterile 35 mm glass bottom Petri dishes (Greiner Bio-one, Germany). The filamentous actin (F-actin) was labeled with rhodamine phalloidin (Molecular Probes, Europe). Fluorescence images were taken using a Zeiss LSM510 Meta inverted confocal microscope (Carl Zeiss, Germany).

2.2. Flow cytometry

Flow cytometric measurements for single non-confluent cells and cells in monolayer were performed on a Cytomics FC500 flow cytometer (Beckman Coulter, USA). Cells were harvested by trypsinization, diluted to 1,000,000 cells/ml in PBS (Amresco, USA), fixed with 4% paraformaldehyde (Fluka, Germany), permeabilized with 0.1% Triton X-100 (Loba Chemie, Austria) in PBS and stained with rhodamine phalloidin (one set of experiments) or Alexa Fluor 488 phalloidin (Molecular Probes, Europe; two sets of experiments). Actin staining was checked by confocal microscopy. Cells without staining were used as negative control. Integral (total signal per cell) forward and sideward scatter intensities and integral green fluorescence intensity for Alexa Fluor 488 phalloidin staining (FL-1 channel) or integral red fluorescence intensity for rhodamine phalloidin staining (FL-3 channel) of the samples were measured. Near 20,000 cells/sample were analyzed.

2.3. Atomic force microscopy and force spectroscopy

The AFM measurements were performed at room temperature using a commercial atomic force microscope Solver Bio (NT-MDT, Russia) combined with an inverted optical microscope (Olympus, Japan). Just before the AFM measurements the growth medium was replaced by PBS. We used silicon nitride triangular cantilevers MSCT-AUHW (former Veeco Instruments, now Bruker, USA) in contact mode for imaging of living cells. The typical curvature radius of the tips is 10 nm, the length, width, and thickness of the cantilevers are 310, 15, and 0.55 μm, respectively, and force constant $k = 0.01$ N/m.

For the force spectroscopy experiments we used the tipless AFM probes CSG11 (NT-MDT, Russia) glued with a 9 μm diameter polystyrene bead. To measure Young's modulus (elastic modulus, stiffness, E) 3–5 force were taken at 1 μm/s rate on each of the studied cells near its center, where the cell height is large enough to apply the Hertz model for data processing. The elastic moduli obtained from these curves were averaged to give the E value for each cell. For each of the non-confluent cell states (formed by seeding or by wound healing assay) three independent experiments were conducted, 15–25 cells were analyzed in each experiment. During experiments with the cell monolayer 15–20 force curves were taken on each of 4–5 areas (400–500 μm diameter), because of the rather small cell projection area. Hertz's contact model was applied for data processing (see Supplementary information for details). To compare the mechanical properties of cells in different states, E was measured at fixed indentation speed (1 μm/s) and at fixed indentation depth (500 nm).

2.4. Analysis of cell viscoelastic properties

To characterize the viscoelastic behavior of cells we measured their mechanical response at varying indentation rates (0.25–8 μm/s), as described in the work by Nawaz et al. (2012). Due to significant difference between the cantilever spring constant and the cellular spring constant we admitted that the cell indentation speed is identical to the piezo movement speed.

Besides, we measured the loss tangents (or hysteresivity) of cells. Sinusoidal perturbation with small amplitude (less than 20 nm) and low frequency (0.3 Hz) was added to the vertical scan control voltage after indentation to 500 nm depth. The cantilever deflection (stress) and scanner Z position (strain) were sampled at ~200 Hz for 50 s. The Fast Fourier transform analyses were used to determine phase shift between stress and strain (Alcaraz et al., 2003; Rico et al., 2005). When calculated this way, η does not depend on k, v and R and other uncertainties associated with Hertzian contact mechanics (Alcaraz et al., 2003). The calculated loss tangent (tangent of the phase shift) provides a measure of solidlike ($\eta < 1$) or fluidlike ($\eta > 1$) behavior of the cell. Given the low oscillatory frequency applied (0.3 Hz), we neglected the hydrodynamic drag influence (Alcaraz et al., 2002). At least 10 cells in each state were analyzed to obtain the indentation rate dependences of E and η. Data were processed with Origin software (OriginLab Corp., Northampton, MA, USA). All AFM experiments were performed during a 30–40 min period. Cell detachment and viability decrease started only after 3–4 h as determined by the trypan blue exclusion test (Strober, 2001).

2.5. Statistical analysis

The non-parametric Mann–Whitney U test was used to determine if differences existed between the groups. The Shapiro–Wilk test was used for the normality test. Analysis was performed using Statistica software, version 8.0 (StatSoft, USA). Young's modulus values were log-normally distributed (meaning that the logarithms were normally distributed) and thus were described using geometric means and multiplicative standard deviations (see below).

3. Results

3.1. Morphology and cytoskeleton structure of Vero cells in different states

The single cells were prepared either by seeding or by wound healing assay, when cells migrated into the scratch wound from

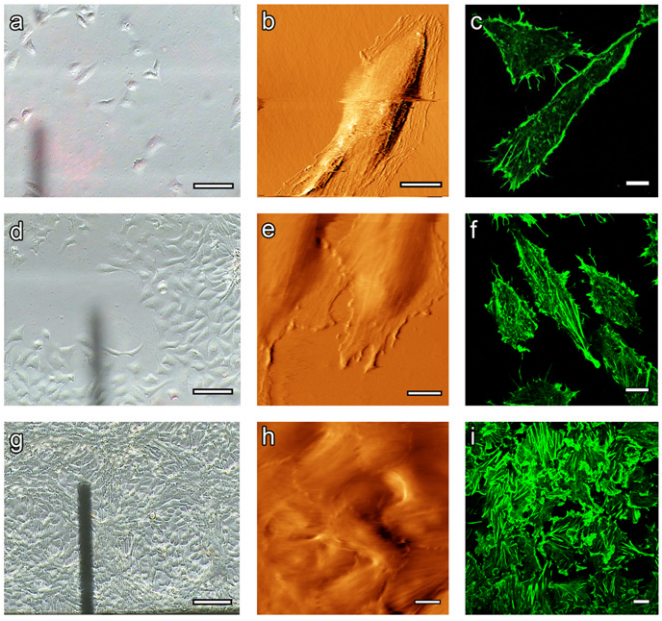

Fig. 1. Bright field images (a, d, and g), AFM deflection images (b, e, and h) and confocal images (c, f, and i) of Vero cells. (a–c) Single cells, (d–f) cells in wound, and (g–i) cells in monolayer. The shadow of the cantilever is seen on the bright field images. Scale bar represents 100 μm on bright field images and 10 μm on the others.