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Exploring the elasticity and adhesion behavior of cardiac fibroblasts by atomic force microscopy indentation

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ABSTRACT

AFM was used to collect the whole force–deformation cell curves. They provide both the elasticity and adhesion behavior of mouse primary cardiac fibroblasts. To confirm the hypothesis that a link exists between the membrane receptors and the cytoskeletal filaments causing therefore changing in both elasticity and adhesion behavior, actin-destabilizing Cytochalsin D was administrated to the fibroblasts. From immunofluorescence observation and AFM loading/unloading curves, cytoskeletal reorganization as well as a change in the elasticity and adhesion was indeed observed.

Elasticity of control fibroblasts is three times higher than that for fibroblasts treated with 0.5 μM Cytochalasin. Moreover, AFM loading–unloading curves clearly show the different mechanical behavior of the two different cells analyzed: (i) for control cells the AFM cantilever rises during the dwell time while cells with Cytochalasin fail to show such an active resistance; (ii) the maximum force to deform control cells is quite higher and as far as adhesion is concern (iii) the maximum separation force, detachment area and the detachment process time are much larger for control compared to the Cytochalasin treated cells. Therefore, alterations in the cytoskeleton suggest that a link must exist between the membrane receptors and the cytoskeletal filaments beneath the cellular surface and inhibition of actin polymerization has effects on the whole cell mechanical behavior as well as adhesion.

1. Introduction

Like bubbles, a membrane that separates the contents from the outside environment characterizes cells. However, there are major dissimilarities: the cell interior contains the cytoskeleton, a gel composed of cross-linked, long-chain proteins; this provides the cell with shear rigidity and shape integrity. The cytoskeleton contains several structures: actin, microtubules and intermediate filaments, each of which has different elastic properties [1].

Furthermore, the cell shape is regulated by several factors, among them (i) the cell proliferation and metabolism, (ii) the internal forces generated by the cytoskeleton, (iii) cell motility, and (iv) the properties of the extra-cellular matrix (ECM). For instance, there are examples of curved membrane proteins responsible for inducing concave or convex curvature [2]. Moreover, these membrane proteins are known to promote actin polymerization that can induce the spontaneous initiation of membrane protrusions [3,4]. The cell membrane curvature is therefore crucial since it finalizes the connection between the membrane shape and membrane protein density, allowing an increase in cytoskeletal forces acting on the membrane and eventually promoting new cell protrusions. It is has also been shown that the membrane adhesion molecules such as integrins aggregate at regions of high convex membrane curvature [5–9]. Cell adhesion to the extracellular matrix (ECM) is mediated by integrins, proteins that regulate and couple the ECM to the actin cytoskeleton filaments [10]. These adhesion membrane proteins are therefore accountable for the recruitment of actin polymerization to the membrane and to the membrane tension due to the adhesion with the extracellular matrix or from the force of actin polymerization, and both forces regulate the cell shape. Furthermore, the ECM–integrin–actin "bridge" provides an important physical connection between the ECM and cytoskeleton for bi-directionally transducing external forces into biochemical signals and forces from the cytoskeleton to the extracellular environment [11,12].

Alterations in the cell adhesive properties trigger numerous pathologies and disease processes such as in metastasic diffusion [13–16], and muscular dystrophies [17,18].

Atomic force microscopy (AFM) is particularly useful in studying interactions between biological molecules since it allows molecular resolution imaging in aqueous media [19,20]. In particular it is well suited for studying the evolution of adhesion forces between the AFM tip (normally a microsphere) and the cell membrane. Typically in this test, the cells are plated on the substrate and in contact with the cantilever only during force measurement. The AFM indenter is moved toward the cell

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membrane, comes into contact with it, and the microsphere is allowed to adhere on the cell membrane during the holding period. Upon retraction, the AFM sphere adheres to the cell membrane and causes opposite deflections of the probe compared to the loading process; therefore the adhesion force can be assessed during the unloading cycle. Such events are typically related to protein unfolding [21] and receptor–ligand binding [22]. Considerations about adhesion and detachment forces such as force steps and tether extraction forces might also be evaluated from unloading curves. Furthermore, the area between the loading and unloading curves reflects viscoelastic cell hysteresis. Based on these considerations, it is our belief that the study of whole-cell AFM force–deformation curves, during a loading and unloading cycle, provides more a comprehensive insight into cell biomechanical behavior.

In particular, this study focuses on the cardiac fibroblast, the cell responsible for the structural integrity of the heart but also responsible for the pathologic myocardial fibrosis commonly found in association with cardiac hypertrophy, cardiomyopathy and failure. Fibrosis results in cell morphology changes, cytoskeletal alterations, and overall in changes in the bulk mechanical properties of the myocardial tissue [23,24]. In cardiomyopathy cytoskeletal proteins mutations could trigger this pathology, involving alterations of the membrane-associated proteins [25,26].

In this paper, the elasticity, viscoelasticity and adhesion behavior of mouse primary cardiac fibroblasts by AFM indentation has been measured. The main focus of this study was to investigate cell elasticity, cell adhesion and it relationship with cytoskeletal organization. The capability of AFM to simultaneously measure cellular mechanical properties and adhesion forces makes it an ideal technique to test the hypothesis that adhesion behavior is related to the integrin density, cytoskeletal alterations, and changes in the cell elasticity and therefore that membrane receptors that are physically connected with cytoskeletal elements serve as a link between the external mechanical environment and the internal signaling of the cell. To understand to what extent the adhesion as well as elastic properties are linked and triggered by parts of the cytoskeleton, we used Cytochalasin D to alter the polymerization kinetics of the actin cytoskeleton filaments.

AFM force–deformation curve method is already used to measure cell elasticity and the use of drugs like Cytochalsin to chemically disassemble the actin network is also well known; for instance, Rotsch et al. [27] found that cultured rat liver macrophages treated with this drug have an average elastic modulus seven-fold less than control cells. However, this paper emphasizes that the whole force–deformation curve carries several information: (1) the total force required to deformed the nucleus, (2) the AFM cantilever deformation at the holding point, (3) the hysteresis area between the loading and unloading cycle, and (4) the area under the deformation curves during the unloading cycle which reflects the cell adhesion behavior. Even if every piece of information is well known in general, it is normally used as a single evidence. In our case we correlate all information to the mechanical properties as well as the adhesion behavior of very interesting cells like cardiac fibroblasts.

2. Materials and methods

2.1. Culture of adult cardiac fibroblasts

Primary expanded culture of cardiac fibroblast was established from adult C57 mice in agreement to institutional guidelines and in compliance with national and international laws and policies (European Economic Community Council Directive 86/609, OJL 358, December 12, 1987). Briefly, whole hearts were extracted, rinsed with CBFHH (Calcium and Bicarbonate Free Hanks with Hepes, composition in mM: NaCl 137; KCl 5.36; Mg_2SO_4 0.81; Dextrose 5.55; KH_2PO_4 0.44; Na_2HPO_4 $7H_2O$ 0.34; Hepes 20.04; pH = 7.4) and minced into pieces. Tissue was rinsed twice with a digesting solution (Trypsin 1.75 mg/mL and DNAase 10 μg/mL in CBFHH) and supernatant was discarded. Every cycle of tryptic digestion was performed placing tissue suspension on a stir plate for 10 min and gently pipetting up and down the tissue 10 times. To ensure maximal collection of fibroblasts at least 8–10 digestion cycles were performed. At the end of the digestion cells were centrifuged (1500 rpm, 10 min, RT). Cells re-suspended in a complete medium (DMEM Glutamax high glucose 4.5 g/l and 20 % Fetal Bovine Serum, Gibco) and kept in culture at sub-confluent density and seeded according to experimental need.

2.2. Immunofluorescence

Cells grown on p35 plates (Falcon, Becton Dickinson, USA) were fixed in PBS containing 3.5% PFA for 20 min; aldehydes were quenched with 0.1 M glycine in PBS for 10 min at room temperature. Cells were permeabilized with 1% Triton X-100 for 30 min, and blocked with 2% BSA and 0.05% sodium azide in PBS (blocking buffer) for 1 h at room temperature. F-actin filaments were labeled with FITC fluorescent conjugated Phalloidin for 1 h at room temperature. (1:20, P5282, SIGMA). Cells were then washed three times for 10 min with PBS and 0.05% Tween 20. Samples were mounted in Vectashield plus DAPI to stain the nuclei (Vector Laboratories).

2.3. AFM measurements

An AFM Solver Pro-M (NT-MDT, Moscow, Russia) was used to acquire morphology as well as force–displacement curves. The AFM was equipped with a "liquid cell" setup with a standard cantilever holder cell for operating in liquid at controlled temperature. Commercially available cantilevers having tips of polystyrene microsphere (diameter about 10 μm by scanning electron microscopy imaging) coated with a gold layer were used (PNP-DB, Nanoworld, Neuchatel, Switzerland). The cantilever force constant was calibrated using the thermal fluctuation method. For soft biological samples it is suggested to use spherical probes since the force is applied to a broader cell area than would be the case if a sharp tip is used, resulting in a lower pressure and less cell damage. But this is not the only reason to prefer spherical indenters. Cells or tissues are very inhomogeneous, consisting of different components (nucleus, cytoskeletal components, etc.); therefore a sphere tip will return better data for such inhomogeneous materials. AFM probes were cleaned, prior to the indentation experiments, by submerging them successively in ethanol and chloroform (30 min each), in order to remove contaminant molecules adsorbed on the probe surface. All studies were performed on living, intact cells in a cell culture medium. Only well-spread and isolated cells were investigated. Those with a round shape and a dark edge were rejected. The basic AFM technique for quantitative analysis of the cell elasticity is the force spectroscopy (called force–curve analysis). The relation between displacement and indentation of the cantilever in contact with the cell was obtained on advancing and retracting curves, called force curves representing the loading and unloading force. Force curves were collected by monitoring cantilever deflection while moving the piezoscanner resulting in a plot of force versus sample position (see Fig. 1). Indentation depth is calculated comparing the curve detected on the glass substrate and the curve recorded on the cell. To calibrate the cantilever deflection signal, curves of force versus the piezo displacement were acquired on the hard substrate of the cells (glass). The AFM tip was moved toward the cell with speeds of 0.5 μm/s. The speed range was chosen to avoid cell movement (at low compression speed) or hydrodynamic force contribution (significant at high speed). To minimize the possible damage to the cell membrane and contamination of the AFM tip, the cells were not scanned before the indentation experiments. Measurements performed around the nucleus are less affected by artifact due to the substrate stiffness [28]. The distal regions, away from the nucleus, were therefore avoided. For each experimental condition, at least 18 cell data are collected, 4 force curves are acquired for every cell, making a grid around the nucleus. In the case of cells treated with Cytochalasin D only 2 force curves are acquired for every cell; however more cells have been tested to acquire the same curve number. These data were enough to detect statistically