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Cell shape-dependent early responses of fibroblasts to cyclic strain

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ABSTRACT

Randomly spread fibroblasts on fibronectin-coated elastomeric membranes respond to cyclic strain by a varying degree of focal adhesion assembly and actin reorganization. We speculated that the individual shape of the cells, which is linked to cytoskeletal structure and pre-stress, might tune these integrin-dependent mechanotransduction events. To this aim, fibronectin circles, squares and rectangles of identical surface area (2000 μm^2) were micro-contact printed onto elastomeric substrates. Fibroblasts plated on these patterns occupied the corresponding shapes. Cyclic 10% equibiaxial strain was applied to patterned cells for 30 min, and changes in cytoskeleton and cell-matrix adhesions were quantified after fluorescence staining. After strain, megakaryocytic leukemia-1 protein translocated to the nucleus in most cells, indicating efficient RhoA activation independently of cell shape. However, circular and square cells (with radial symmetry) showed a significantly greater increase in the number of actin stress fibers and vinculin-positive focal adhesions after cyclic strain than rectangular (bipolar) cells of identical size. Conversely, cyclic strain induced larger changes in pY397-FAK positive focal complexes and zyxin relocation from focal adhesions to stress fibers in bipolar compared to symmetric cells. Thus, radially symmetric cells responded to cyclic strain with a larger increase in assembly, whereas bipolar cells reacted with more pronounced reorganization of actin stress fibers and matrix contacts. We conclude that integrin-mediated responses to external mechanical strain are differentially modulated in cells that have the same spreading area but different geometries, and do not only depend on mere cell size.

1. Introduction

Cells within tissues are under tight regulation of the extracellular matrix (ECM), which provides an exoskeleton for them to attach, to move around, and to perform the functions required for their own survival and to maintain tissue homeostasis [1,2]. Within tissues, cells have to withstand mechanical forces that are exerted by various physiological processes, starting from embryonic development to regular body functions in adults [3,4]; a prominent example is the morphogenesis, mechanobiology and repair of tendons [5,6]. Cells sense the strains in the ECM generated by mechanical stresses, and respond to them by remodeling their cytoskeleton and the matrix around them in order to maintain tissue integrity. Many studies have shown how cultured cells react to cyclic strain and shear stress. In order to understand how cells respond to mechanical stimulation, they are grown on ECM-coated elastomeric membranes and subjected to uniaxial or biaxial cyclic strain for various time intervals *in vitro*. Fibroblasts, endothelial, and smooth muscle cells are favored for such studies; uniaxial strain causes the actin cytoskeleton to reorganize perpendicular to the direction of stress [7], whereas a reverse pattern is seen in endothelial cells subjected to fluid shear stress, which reorganize their cytoskeleton parallel to the flow direction [8,9].

Changes in the actin cytoskeleton in response to external strain depend on integrin and RhoA signaling [10–13]. Cell matrix adhesions are the expected sites where force is transmitted from ECM to cytoskeleton [14]. During cell adhesion, first contact with ECM leads to recruitment of integrins at these sites, primarily classified as focal complexes [14]. The clustering of integrins is linked to recruitment of focal adhesion kinase (FAK) at the cell periphery [15]. Focal adhesion kinase is not required for the formation of matrix adhesions, but is essential for their dynamics and turnover [16]. FAK is activated by autophosphorylation at Tyr397, either upon integrin clustering in

Abbreviations: BSA, Bovine Serum Albumin; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Medium; ECM, extracellular matrix; PBS, phosphate-buffered saline; FAK, focal adhesion kinase; FCS, fetal calf serum; HMDS, hexamethyldisiloxane; LIM, *lin-11, Isl-1* and *mec-3*; MEFs, mouse embryonic fibroblasts; MKL-1, megakaryocytic leukemia-1; PDMS, polydimethylsiloxane; RhoA, Ras homolog gene family, member A; ROCK, Rho-associated Protein Kinase; SRF, serum response factor; TRITC, tetramethylrhodamine isothiocyanate

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newly forming focal complexes [15], or in response to tensile strain acting on matrix adhesions [17]. Inhibition of RhoA/ROCK before applying strain abolishes FAK phosphorylation [18]. Tensile strain-induced FAK phosphorylation leads to the activation of Rac1 and the formation of lamellipodia [15], and FAK null fibroblasts fail to respond to mechanical cues by cell reorientation [7]. External and/or cellular forces are required for RhoA/mDia1 dependent maturation of transient focal complexes into more stable focal adhesions [19], which is characterized by docking of various other focal adhesion associated proteins at the adhesion sites such as talin, paxillin, vinculin and tensin [14], and by an increase in membrane–cytoskeleton interactions. The linking of actin filaments with the focal adhesion plaque generates an inward pull, thus activating vinculin by a conformational switch, which helps in further growth and stabilization of the anchorage site [20]. External tensile force activates the small GTPase RhoA leading to changes in actin cytoskeleton dynamics [10,12,21], whereby mDia1 promotes actin assembly, and Rho-dependent kinase ROCK triggers stress fibre contraction [22]. RhoA-mediated F-actin assembly leads to translocation of megakaryoblastic leukamia-1 protein (MKL-1/MRTF-A/MAL) to the nucleus, where it acts as transcriptional co-activator of serum response factor (SRF) [23] or as *bona fide* transcription factor [24] for the expression of specific mechanoresponsive genes. Others and we reported that tensile strain induces nuclear translocation of MKL-1 in a RhoA-dependent manner [13,25,26]; hence this process can be considered an optical readout for RhoA activation in response to external strain.

Actin remodeling in response to external strain involves not only assembly and contraction, but also reorganization and repair. A considerable interest has been focused on mechanosensitive properties of the focal adhesion associated LIM protein, zyxin, in recent years. In resting cells, zyxin is predominantly localized at focal adhesions [27], where it interacts with the stretch-sensing protein p130Cas [28]. This and the fact that zyxin promotes actin polymerization [29] suggest a role in strain-induced reorganization of the actin cytoskeleton [30]. Indeed, uniaxial cyclic strain of high intensity leads to a rapid re-distribution of zyxin from focal adhesions to stress fibers in both fibroblasts and endothelial cells [31]. The protein appears to be crucial for stress fiber stabilization and repair in response to excessive strain, since this process is compromised in zyxin-deficient fibroblasts [32].

We have previously obtained preliminary evidence that the extent of RhoA-dependent responses of fibroblasts to cyclic substrate strain depends on the state of the actin cytoskeleton at the time when the mechanical stimulus is applied [11,12]. When fibroblasts are plated *in vitro* on flat substrates, they assume random shapes and thus are very heterogeneous to each other with respect to how their actin cytoskeleton is structured. This heterogeneity in cytoskeletal arrangements appears to lead to different kinds of responses to external mechanical stimuli, whereby certain fibroblasts assemble many stress fibers and contract heavily upon cyclic strain compared to others [11,12]. The structure of the actin cytoskeleton determines cell shape and *vice-versa* [33], and cytoskeletal pre-stress generated by actomyosin-dependent traction force is known to be correlated with cell shape [34]. We therefore hypothesized that the specific RhoA- dependent responses of an individual cell to external substrate strain depend on its shape and pre-stress at the time when strain is applied.

Surface micro-patterning provides a unique possibility to create uniform cell shapes for *in vitro* culture and to explore the basics behind cell–matrix interactions. Micro-contact printing is a soft lithography technique, which is the most practicable method to apply designed protein patterns on a wide range of culture substrates. Cells adhere on patches printed on the substrate with ECM protein, and adapt their shape accordingly [33,35,36]. To test our hypothesis, we printed fibronectin islands of different shapes – circles, squares, and rectangles – of 1:2 and 1:4 aspect ratio onto silicone elastomer membranes. To rule out effects of different cell size, all fibronectin patches had a fixed area of 2000 μm^2, which allowed extensive spreading of fibroblasts while still confining their shape. Cells attached to these geometries were then subjected to cyclic equibiaxial strain for 30 min at 0.05 Hz on a custom-made device [37]. Changes in MKL-1 distribution, cell–matrix adhesions, and the actin cytoskeleton were monitored by immunofluorescence methods. The results obtained from this study indicate that RhoA-mediated responses to cyclic strain differ in magnitude as well as quality depending on whether a cell has a radially symmetric or bipolar shape.

2. Materials and methods

2.1. Photolithography

We designed a silicon master containing repetitive fields of desired geometries and dimensions by conventional photolithography process. The silicon wafer was primed with hexamethyldisiloxane (HMDS) followed by spin coating a 1.5 μm thick layer of AZ® 1512 (MicroChemicals GmbH, Ulm, DE) positive photoresist at 3000 rpm for 90 s. The wafer was then soft baked at 112° C for 90 s and the micro-patterns were transferred on the resist with the i-line of an Hg lamp through a Cr mask at a dose of 90 mJ/cm^2 using an MJB4 mask aligner (SÜSS MicroTec, Garching, Germany). The exposed resist layer was developed for 55 s in 100 ml of MicropositTMMFTMCD-26 developer (Shipley, Marlborough, MA, USA). The desired pattern was finally transferred to the silicon wafer and etched to a depth of 15 μm by a BOSCH etching process using SF6 and C4F8 plasma (Alcatel 601 E, AMMS, Annecy, France). The remaining photoresist was stripped with an oxygen plasma cleaner for 7 min (Tepla 300, PVA TePla, Kirchheim, Germany). The final master has fields of circular, square, and rectangular (1:2 and 1:4) wells of 2000 μm^2 in area etched in it as summarized in Fig. 1A. Before using the Si master as a mold for the production of PDMS stamps, it was silanized by placing it overnight in a vacuum desiccator along with few drops of trichloro-(1H,1H,2H,2H-perfluorooctyl)-silane (Sigma–Aldrich GmbH, Buchs, Switzerland).

2.2. Micro-contact printing

The micro-contact printing was carried out by a conventional protocol used before in several studies [38]. The elastomeric PDMS stamps were made out of Sylgard 184 (Dow Corning, Seneffe, Belgium) by mixing base and cross-linker at ratio 1:10 (w/w) and rendered hydrophilic by treating with air plasma for 30 s using a portable corona-system ('Plasma Pen'- model BD20V; Electro-Technic Products Inc., Chicago, Illinois, USA). The stamps were 'inked' with 50 μg/ml gelatin-affinity purified horse serum fibronectin [26], which in some experiments was labeled with Alexa Fluor® 350 (Molecular Probes; Life Technologies, LuBioScience, Lucerne, Switzerland) for 1 h. The fibronectin solution was removed and the surface was dried with a flow of nitrogen. The desired geometries were then printed on stretchable silicone membrane (0.05", gloss/gloss; Specialty Manufacturing Inc., Saginaw MI, USA). To obtain reliable printing, it was important to previously render the silicone membrane hydrophilic by plasma treatment (see above). This printed membrane was then mounted on a custom-made stretching device [37] and the surrounding area of fibronectin patterns was blocked by 1% bovine albumin serum (BSA; Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM Na-phosphate, pH 7.4) to prevent non-specific cell adhesion. The substrate was rinsed with PBS and serum free Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Life Technologies, LuBioScience, Lucerne, Switzerland) before plating of cells.

2.3. Cell culture and mechanical stressing

A clonal mouse embryonic kidney fibroblast cell line (MEFs) was used for all experiments [26]. Cells were maintained at 37 °C in