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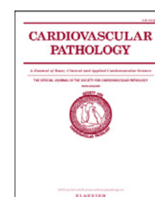

Original Article

Substrate stiffness affects sarcomere and costamere structure and electrophysiological function of isolated adult cardiomyocytes

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ABSTRACT

Introduction: The mechanical environment is a key regulator of function in cardiomyocytes. We studied the role of substrate stiffness on the organization of sarcomeres and costameres in adult rat cardiomyocytes and further examined the resulting changes in cell shortening and calcium dynamics.
Methods: Cardiomyocytes isolated from adult rats were plated on laminin-coated polydimethylsiloxane substrates of defined stiffness (255 kPa, 117 kPa, 27 kPa, and 7 kPa) for 48 h. Levels of α-actinin and β1 integrins were determined by immunofluoresence imaging and immunoblotting, both in the absence and presence of the phosphatase inhibitor calyculin A. Quantitative reverse transcriptase polymerase chain reaction was used to measure message levels of key structural proteins (α-actinin, α7 integrin, β1 integrin, vinculin). Sarcomere shortening and calcium dynamics were measured at 2, 24, and 48 h.
Results: Overall cardiomyocyte morphology was similar on all substrates. However, well organized sarcomere structures were observed on only the stiffest (255 kPa) and the most compliant (7 kPa) substrates. Levels of α-actinin in cells were the same on all substrates, while message levels of structural proteins were up-regulated on substrates of intermediate stiffness. Inhibition of phosphatase activity blocked the degradation of contractile structures, but altered overall cardiomyocyte morphology. Shortening and calcium dynamics also were dependent on substrate stiffness; however, there was no clear causative relationship between the phenomena.
Conclusions: Extracellular matrix stiffness can affect structural remodeling by adult cardiomyocytes, and the resulting contractile activity. These findings illuminate changes in cardiomyocyte function in cardiac fibrosis, and may suggest cardiac-specific phosphatases as a target for therapeutic intervention
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1. Introduction

Substrates with tunable stiffness provide crucial information about how cells sense and respond to the mechanics of their environment. Modulation of the mechanical environment has been used with substantial success for controlling stem cell fate [1], discerning the contractility of smooth muscle cells and other mesenchymal cell types [2,3], and observing the response of neonatal cardiomyocytes to the stiffness environment of the developing heart [4–12]. However, no studies have directly observed the effect of substrate stiffness on adult cardiomyocytes. In vivo, these cells secrete a basement membrane containing laminin and collagen IV that promotes integration with the myocardial extracellular matrix and provides a mechanically supportive network. There is growing interest in understanding how the extracellular microenvironment and, in particular, the mechanical environment, affects cardiomyocyte function. A main goal of this research is to identify the mechanisms by which cell–matrix interactions malfunction in pathology so that treatments can be developed to prevent progression to heart failure. The present study uses substrates of tunable stiffness to study the effects of extracellular matrix compliance on sarcomere and costamere organization, as well as contraction dynamics, in adult cardiomyocytes.

The question of how cardiomyocytes sense strain is crucial to understanding how these cells regulate their contraction/relaxation dynamics during systole and diastole, and multiple studies have identified proteins associated with a “stretch sensor” mechanism [13,14]. However, it is likely that cardiomyocytes also have a “stress sensor” that can react to the changes in myocardial stiffness that occur as a result of pathology (myocardial infarction, hypertrophic cardiomyopathies, etc.) and aging. Atomic force microscopy measurements of the elastic modulus of fibrotic myocardium have yielded values between 20 and 55 kPa [15,16], compared to 2 and 8 kPa for nonfibrotic myocardium as determined by magnetic resonance imaging [17]. Though it can be difficult to quantitatively compare elastic moduli determined by different methods [18], these data

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suggest that increased deposition of extracellular matrix in fibrotic tissue results in a mechanically stiffer microenvironment. A recent study indicated that substrate stiffness resembling a fibrotic scar inhibited beating of embryonic cardiomyocytes [19].

Proteins involved in sensing of stretch have been identified in both the sarcomere and the costamere complex [13]. The function of the costamere is to couple the z-disk to the basement membrane so that force generated by the sarcomere can be translated to the surrounding extracellular matrix. The costamere complex includes β1 and α7 integrins that span the plasma membrane [20]. The β1 integrins in particular are important surface receptors in cardiomyocytes, regulating cell–matrix interactions [21] and playing a role in cytoprotection [22]. Integrins are anchored to the z-disk by structural proteins including vinculin, talin, desmin, and zyxin [14]. The z-disk complex is a complicated array of proteins that also includes candidates for the stretch-sensing mechanism, with α-actinin forming the backbone of the structure. Recent studies have indicated that the z-disk is among the initial targets for deterioration during isoproterenol-induced adult cardiomyocyte apoptosis [23], suggesting that z-disk integrity is a crucial indicator of cardiomyocyte function.

In the present study, components of both costamere and sarcomere structure and function in culture were analyzed in response to changes in substrate stiffness. Adult cardiomyocytes in culture beat spontaneously and undergo a slow dedifferentiation process, which involves changes in the cytoskeleton and t-tubule structure [24,25]. The calcium handling in these cells also changes with time, possibly due to influx through protein kinase A-activated L-type Ca^{2+} channels [26]. To determine how the stiffness of the extracellular environment affects these properties, isolated cardiomyocytes were plated on laminin-coated substrates with varying stiffness and cultured for 48 h. The organization of α-actinin and integrin β1 was observed by immunofluoresence, and message levels of proteins associated with the sarcomere and costamere were measured. The effect of phosphatase inhibition on cytoskeletal rearrangement was also examined using calyculin A, a general phosphatase inhibitor. Finally, cells were paced at different time points to determine how changes in cell structure affected the electrical function of the cardiomyocytes, as well as to characterize the time dependence in the cellular response.

2. Methods

2.1. Cell isolation and culture

Adult rat ventricular cardiomyocytes were isolated as previously described [27]. Briefly, hearts from Sprague–Dawley rats were enzymatically digested in a modified Langendorff perfusion apparatus to isolate the cardiomyocytes. The protocol was approved by The University of Michigan University Committee on Use and Care of Animals in accordance with University guidelines. Aliquots of 2.0×10^4 cells were plated on polydimethylsiloxane (PDMS) substrates coated with 40 μg/mL of laminin, using M199 culture medium supplemented with 5% fetal bovine serum, 50-U/mL penicillin, and 50-μg/mL streptomycin. After 2 h, this medium was replaced with serum-free M199 supplemented with 10-mM HEPES, 0.2-mg/ml bovine serum albumin, 10-mM glutathione, 50-U/mL penicillin, and-50 μg/mL streptomycin. Cells were cultured in standard incubators with 5% CO_2 at 37°C. For experiments involving calyculin A, 0.5 μg/mL of the phosphatase inhibitor was added to the media. Different cells on separate substrates were used for the immunofluorescence, sarcomere shortening, and calcium transient assays.

2.2. PDMS substrate fabrication and validation

The base and curing agent (Sylgard 184) were mixed in ratios of 10:1, 20:1, 30:1, and 50:1 by weight, and 1.0 mL of the elastomer was cured in 6-well polystyrene plates, creating slab constructs approximately 1 mm thick. For experiments that required electric field stimulation, blocks of the elastomer were cut out of the 6-well plate and bonded to glass coverslips. Prior to coating with laminin, the PDMS was etched with 5-M sulfuric acid for 60–90 min, washed thoroughly in distilled water, and sterilized using an ultraviolet lamp for 30 min. To determine elastic modulus, the elastomer was molded in a rectangular mold with defined width and length. A uniaxial mechanical testing system was used to apply 20% total strain at a rate of 10 %/s. The elastic modulus was calculated as the slope of the stress/strain curve at approximately 10% strain, in order to keep within the limits of linear strain theory. PDMS mixed in ratios of 10:1, 20:1, 30:1, and 50:1 produced substrates with mean moduli of 255 kPa, 117 kPa, 27 kPa, and 7 kPa, respectively.

2.3. Immunofluoresence

After 48 h in culture, cells were fixed, permeabilized, and stained using previously described protocols [3]. Immunostains included fluorescent DAPI (1:50 dilution), phalloidin (1:50) conjugated to Alexa Fluor 488, and monoclonal mouse antibodies for α-actinin (1:500) and integrin β1 (1:50). A secondary Texas Red-tagged anti-mouse antibody was used to visualize these proteins. Cells were visualized using confocal microscopy. Projection image Z-stacks of approximately 10 μm were collected using a confocal microscope.

2.4. Immunoblotting

After 48 h in culture, cells were lysed in ice-cold sample buffer (24.77% glycerol, 1.63% SDS, 0.1 M Trizma stacking buffer, 3.31% Bromophenol Blue stock solution, 24.88-mM DTT, 1.78-mM leupeptin) and then stored at −20°C. In preparation for protein separation, samples were boiled for 3 min and then sonicated for 10 min. Protein separation was performed as previously described [27] using 12% or 4–12% gradient sodium dodecyl sulfate–polyacrylimide 12-well gels (Bio-Rad) and then transferred onto a PVDF membrane overnight. The PVDF membrane was then blocked in either 5% dry nonfat milk or bovine serum albumin, washed in Tris-buffered saline (TBS), and incubated overnight at 4°C with a mouse monoclonal antibody against α-actinin (1:5000). The next day, blots were rinsed with TBS and incubated for 45 min at room temperature with horseradish peroxidase–linked anti-mouse secondary antibodies (1:2000). Gels were silver-stained, and a representative protein band was used to normalize for protein loading on the blot. Immunodetection was visualized with chemiluminescence using Pierce ECL Western Blotting substrate (Thermo-Fisher).

2.5. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

A guanidium thiocyanate-phenol-chloroform extraction protocol (TRIzol) was used to isolate mRNA from the cells after 48 h in culture. Briefly, cells were dissolved in TRIzol and buffer, and reverse transcription of mRNA was performed with a high-capacity cDNA Archive Kit and a C-1000 Thermocycler. The complete quantitative PCR protocol is described in a previous publication [3].

2.6. Cell shortening assay

At 2, 24, and 48 h, PDMS coverslips were transferred to a stimulation chamber mounted on a microscope stage and perfused with M199 culture media at 37°C throughout the assay. A video-based detection system (Ionoptix) was used to determine sarcomere shortening in response to 40V, 0.2 Hz electrical shortening.

2.7. Calcium transient assay

At 2, 24, and 48 h, cells on PDMS coverslips were loaded with 5.0-μM Fura-2 AM for 4.5 min at 37°C followed by a 4-min wash in

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