Journal of Biomechanics 46 (2013) 1967–1971

Contents lists available at SciVerse ScienceDirect

Journal of Biomechanics

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

Short communication

Determination of local and global elastic moduli of valve interstitial cells cultured on soft substrates

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ARTICLE INFO

Article history:
Accepted 2 May 2013

Keywords:
Atomic force microscopy
Cell mechanics
Substrate elasticity
Heart valve

ABSTRACT

The elasticity of the extracellular matrix profoundly affects biological responses of cells, but also their mechanical properties. Single cell mechanical properties are often measured by atomic force microscopy (AFM), but technical guidelines for AFM measurement of cells grown on soft substrates are not well established. In this study, the local and global elastic moduli of aortic valve interstitial cells (VICs) cultured on soft polyacrylamide substrates (3–144 kPa) were characterized via AFM force mapping using pyramidal and spherical tips, respectively. Local and global VIC modulus values both increased with substrate stiffness ($p < 0.05$), with the average local cell modulus being consistently two to three times greater than the global modulus ($p < 0.05$). For local measurements, a minimum of four measurements was required to observe the trend of increasing cell modulus with substrate stiffness, but there was no advantage to testing additional spots. The Hertz model was relatively accurate in estimating the global cell elastic modulus (< 12% error, based on validated finite element analyses), despite the cells being of finite thickness and grown on deformable substrates, neither of which are accounted for in the Hertz model. The results of this study provide practical guidelines for efficient AFM-based measurement of the mechanical properties of cells on gels. They also provide new physiologically-relevant data on VIC mechanical properties and their correlation with substrate stiffness-dependent cytoskeletal changes, with relevance to heart valve mechanobiology and disease.

1. Introduction

The cellular mechanical environment profoundly affects many cellular mechanical and biological responses. Force transfer from the extracellular matrix (ECM) to an attached cell depends on the inherent mechanical properties of both of the ECM and cell (Georges and Janmey, 2005). Importantly, some cells can adapt their stiffness to that of their substrate (Solon et al., 2007), creating a complex, reciprocal relationship between cell mechanics, ECM mechanics, and cell mechanobiological responses. Because of the critical roles played by cell and ECM mechanics in several diseases (Chen and Simmons, 2011b), there is much interest in understanding how cell mechanical properties are influenced by ECM mechanics.

The mechanical properties of single adherent cells are often measured by atomic force microscopy (AFM) (Azeloglu and Costa, 2011; Mathur et al., 2001), which is capable of both local and global measurements, can measure forces that span orders of magnitude (μN–pN), and is immune from potential heat generation as in magnetic bead microrheometry and photodamage to cells as in optical tweezers.

In AFM, cells are typically indented either with tips mounted with a microsphere to yield a single (global) value for cell elasticity or with conventional pyramidal tips to map local elasticity with high spatial resolution (i.e., force mapping). Young's modulus of the cell is derived from the indentation force and displacement measurements based on the Hertz contact theory, in which the cell is modeled as a half-space with an infinite thickness (Sneddon, 1965). In reality, cells have finite thickness and traditionally have been measured while attached to stiff glass substrates, which can cause overestimation of the cell Young's modulus. Guidelines to mitigate the effects of finite cell thickness and detection of the underlying substrate have been proposed for cells on glass (Costa and Yin, 1999; Dimitriadis et al., 2002; Mahaffy et al., 2000; Rosenbluth et al., 2006), but it is not known how measurements based on Hertz contact are influenced by soft substrates with physiologically-relevant elastic moduli. Further, it is not known if substrate stiffness-dependent cell elastic moduli are detectable by both global and local AFM measurement, and what the effect of measurement spatial resolution is on local elasticity determination.

To provide technical guidelines for AFM measurement of cells on gels, we addressed these questions using a combined experimental and computational approach. We used AFM to measure and compare the global and local elastic properties of cells on soft substrates with

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http://dx.doi.org/10.1016/j.jbiomech.2013.05.001

physiologically-relevant elastic moduli and determined the effect of measurement spatial resolution on local elasticity determination. Based on a parametric finite element analysis (FEA), we tested the sensitivity of elasticity measurements of cells on gels to cell height and substrate stiffness. We used primary aortic valve interstitial cells (VICs) as a model cell type, as they are known to respond biologically to substrate stiffness (Chen and Simmons, 2011a) and elastic modulus measurements have reported for them, albeit under non-physiological conditions (Merryman et al., 2009; Merryman et al., 2007; Wyss et al., 2012). Further, changes in extracellular matrix stiffness are a hallmark of calcific aortic valve disease and thought to influence the pathological differentiation of VICs (Yip et al., 2009). Thus, a secondary goal of this study was to measure for the first time the elastic properties of VICs on physiologically-relevant substrates, thereby providing more relevant input data for computational models (Huang et al., 2007) and new insights into valve mechanobiology and disease.

2. Materials and methods

Detailed materials and methods are available in Supplemental material.

2.1. Cell culture

Aortic VICs were isolated from porcine valve leaflets by collagenase digestion as described previously (Yip et al., 2009) and used at passage three. Polyacrylamide (PA) was used as the culture substrate for its mechanical tunability. VICs were grown on PA hydrogels with compressive elastic (Young's) moduli of 3–144 kPa and coated with type I collagen, as described previously (Chen et al., 2011). This range of substrate stiffness is estimated to mimic normal to fibrotic and stenotic valve tissue, based on micromechanical measurements of native porcine aortic valves (Chen et al., 2011; Yip et al., 2009; Zhao et al., 2011). VIC projected spread area was determined as a function of substrate stiffness by the LIVE/DEAD® Viability Assay and digital image analysis. Only individual VICs with projected areas within 95% confidence bounds of the Gaussian fit mean of the population of individual cells for a specific substrate modulus were selected for indentation tests.

2.2. AFM measurement of cell elastic modulus

VICs were tested using a commercial AFM (Bioscope Catalyst, Bruker, Santa Barbara, CA) mounted on an inverted optical microscope (Nikon Eclipse-Ti). Force mapping was accomplished using a pyramidal tip and by combining topography imaging and indentation in an array of spots selected to evenly cover a region of interest on a cell (Radmacher et al., 1996). The heights of the VICs on PA gels were measured from topography imaging as a part of the force mapping and thus were measured on the same population of cells that were indented. In this study, we used a 16×16 array to cover the whole cell and surrounding gel, which typically generated an array of 6×6 to 8×8 effective measurements entirely on the cell body. Global measurements were done with a spherical tip via 'point and shoot' and microscope image registration and overlay (MIRO) at four distinct spots on the cell. Local measurements were made first via force mapping, and then via MIRO in arrays of 1×1 to 8×8 spots selected to evenly cover the cell surface. All the force curves analyzed were captured on locations with a cell height of at least 3 μm, which was determined from the topography of the cells, and the trigger force applied to the cell was consistently 1 nN. All AFM measurements were done in the fluid environment at room temperature. Force curves were fit to the Hertz model to estimate elastic modulus.

2.3. Immunostaining

VICs were immunostained for α-smooth muscle actin (α-SMA) and nuclei using standard protocols. Fluorescent images were analyzed to determine the total number of cells by counting nuclei and the number of cells with α-SMA-positive stress fibers.

2.4. Finite element analysis

To test the sensitivity of AFM cell elastic modulus measurement to cell thickness and substrate stiffness, we performed a parametric FEA to model the AFM indentation experiments in which a spherical tip was used to measure the global elastic modulus of cells on soft gels. Details of the model and analysis are provided in Supplemental material. Using a prescribed force and the indentation predicted by the FEA, we determined the "measured" elastic modulus predicted by the Hertz model ($E_{measured}$), and used the ratio of the measured modulus to the modulus of the cell layer (E_{cell}) to characterize the deviation of the measured modulus from the actual cell modulus. The sensitivity of $E_{measured}/E_{cell}$ to: (i) the ratio of the gel elastic modulus to the cell modulus (E_{gel}/E_{cell}); and (ii) the cell layer thickness were evaluated. The values of the parameters used were: E_{gel}/E_{cell} ratio = 0.5, 1, 10, 50, 100, 500 and 1000; and cell thickness = 6, 8 and 10 μm. FE predictions were validated experimentally using dual-layer polyacrylamide gels to mimic a cell on a gel, as described in Supplemental material.

3. Results

As the underlying substrate stiffness increased from 3 to 144 kPa, the VIC projected area increased and the cell apex height decreased (Fig. 1a). The VIC average local elastic modulus values obtained with pyramidal tips and full mapping increased with increasing substrate stiffness (Fig. 1b; $p < 0.05$).

We compared the average local cell modulus values determined from measurements of 1×1 to 8×8 evenly selected spots. These experiments demonstrated that the number of local measurements significantly affected the average local cell modulus (Fig. 2; $p < 0.04$). The average local modulus calculated from a single spot was greater than that from four spots ($p < 0.02$), but there was no difference in the average local cell modulus when more than four spots were tested ($p > 0.78$). In other words, the

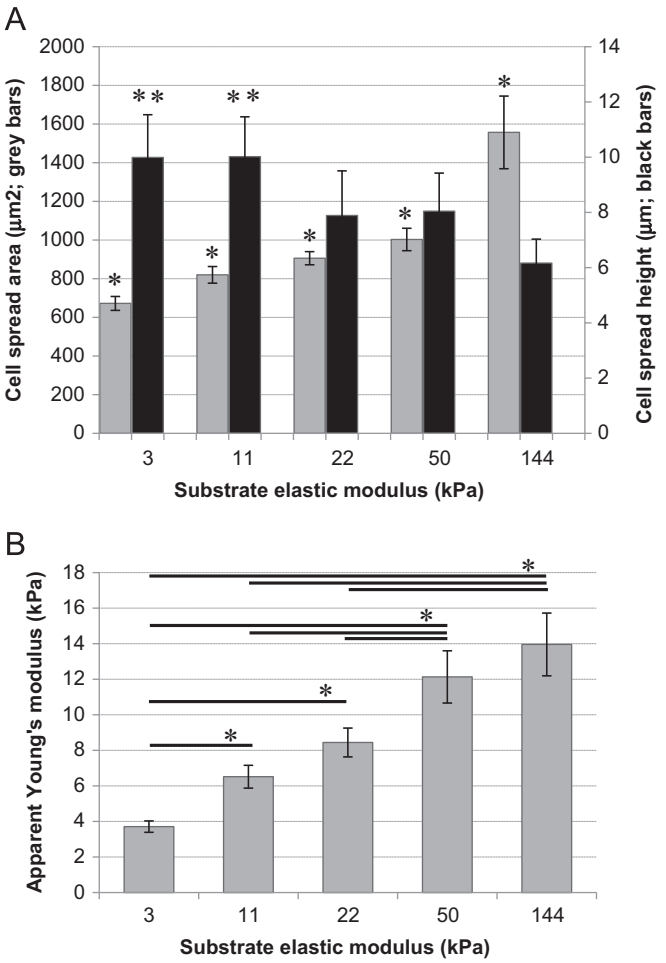

Fig. 1. (A) Spread area (∗$p < 0.05$ versus other substrates; $n = 121$–252) and height (∗∗$p < 0.05$ versus 144 kPa substrate; $n = 10$–12) of cells on different substrate elasticities. (B) Effect of substrate modulus on VIC elastic modulus measured via force mapping with pyramidal tips (average of 6×6 to 8×8 spots) (∗$p < 0.05$; $n = 9$–11).

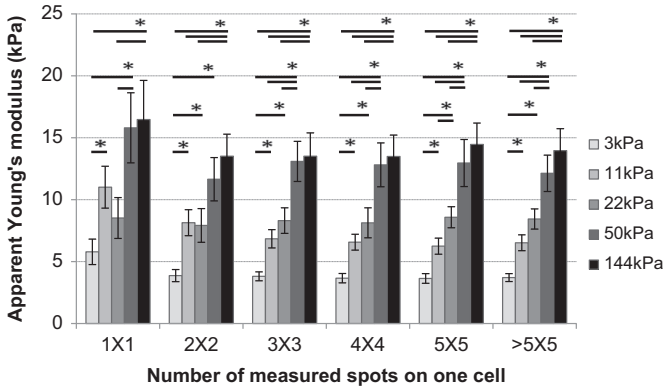

Fig. 2. Comparison of average VIC elastic moduli measured via force mapping with pyramidal tips at different numbers of spots (*$p < 0.05$; n=9–11 for each group).

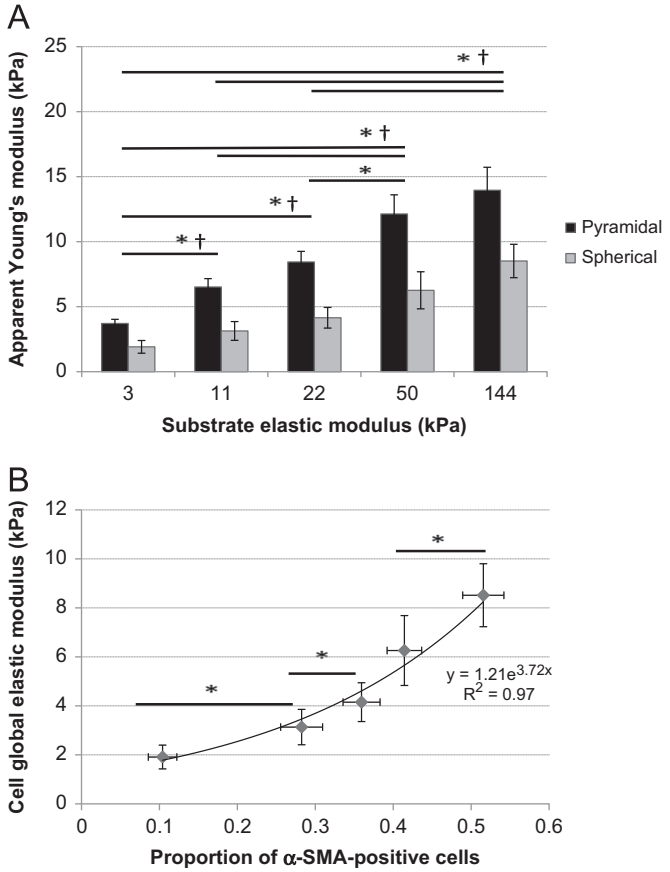

Fig. 3. (A) Effect of substrate modulus on VIC elastic modulus measured via MIRO (point-and-shoot) with spherical tips (†$p < 0.05$; n=9–12) and via force mapping with pyramidal tips (average of 6×6 to 8×8 spots) (*$p < 0.05$; n=9–11); and (B) correlation between the proportion of VICs with α-SMA-positive stress fibers and the cell global elastic modulus measured with spherical tips. (*$p < 0.05$; only some differences shown).

trend of increasing cell modulus with substrate stiffness was observed as long as more than 2×2 spots were tested.

Similar to the local elastic modulus, cell global elastic modulus measured with spherical tips increased with substrate stiffness (Fig. 3a; $p < 0.05$). Notably, the magnitude of the average local cell modulus determined with the pyramidal tip was consistently two to three times higher than the global modulus measured with the spherical tip. The increase in cell global elastic modulus with substrate stiffness correlated positively (R^2=0.97) with a significant increase in α-SMA stress fiber expression (Fig. 3b; $p < 0.05$).

To test the impact of finite cell thickness and the potential deformation of the underlying soft substrate on the cell elastic modulus predicted by the Hertz model, we performed a parametric FEA simulating the spherical tip experiments. We found that the measured modulus was sensitive to both cell height and substrate stiffness, with the greatest errors occurring with the thinnest cells and stiffest substrates (Fig. 4). However, for the E_{gel}/E_{cell} ratios in our experiments (~1.5–16; Fig. 3a), the errors in cell elastic modulus measurements with the Hertz model were predicted to be less than 12%. The model predictions were validated experimentally for two simulated cell heights and a stiff substrate (E_{gel}/E_{cell}=144 kPa/11 kPa=13.1). For 6.1 μm thick top layer gels, the measured modulus of 12.5 ± 0.28 kPa (mean ± standard error) was not statistically different from that predicted by the FEA (12.2 kPa; p=0.30 by one sample t-test). Similarly, the measured modulus for the 7.2 μm thick top layer gels of 11.97 ± 0.24 kPa was not statistically different from that predicted by the FEA (12.0 kPa; p=0.87).

4. Discussion

Guidelines for AFM measurement of the elasticity of cells on soft substrates are lacking. We showed here that (1) global and local AFM measurements can both be used to detect substrate stiffness-dependent changes in cell elastic modulus, but provide different absolute measurements; (2) nine local measurements are enough to characterize the average cell local elastic modulus; and (3) Hertz model estimates of the elastic modulus of cells on soft substrates are minimally affected by cell height and substrate stiffness. This study also provides new physiologically-relevant data on VIC mechanical properties and their correlation with substrate stiffness-dependent cytoskeletal changes.

Measurements with the two AFM tip geometries demonstrated similar trends in the dependency of local and global cell moduli on substrate stiffness. However, the two types of tips yielded different cell modulus magnitudes, and measurements with pyramidal tips consistently produced larger variations in averaged cell moduli. This phenomenon was reported previously (Dimitriadis et al., 2002; Rico et al., 2005) and is often understood to be that smaller modulus values are associated with the larger contact area of spherical tips (Berdyyeva et al., 2005). Additionally, pyramidal tips would be expected to generate large strains locally that would result in hyperelastic responses and make the cells appear stiffer than with spherical tips (Dokukin and Sokolov, 2012). Finally, the

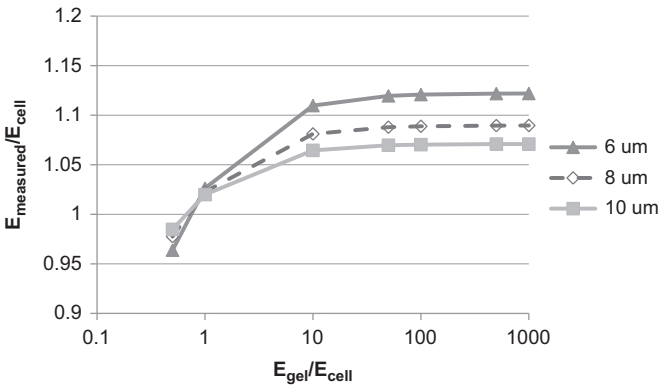

Fig. 4. Sensitivity of cell global elastic modulus measured with spherical tip and the Hertz model to cell height and substrate elastic modulus, as predicted by finite element simulation. For gel substrates with elastic moduli less than that of the cell, the measured elastic modulus is underestimated by up to ~4%, whereas for gels that are stiffer than the cell, the measured elastic modulus is overestimated by up to 12%. The largest errors occur with the thinnest cells. The cell elastic modulus was fixed at 11 kPa for these simulations.

discrepancies between the tips are also likely in part attributable to errors in estimating both the semi-vertical angle of pyramidal tips and radius of curvature of spherical tips. Since the pyramidal tips were not perfectly axis-symmetric (i.e., the tip back angle is often larger than the front angle), this likely resulted in a larger effective conical angle than was used in calculations. An opening angle of a cone that is underestimated by a factor of two results in an overestimation in the Young's modulus by at least 50%, whereas the radius of a sphere that is underestimated by a factor of two results in an overestimation in the Young's modulus by only 29%. This suggests that the Hertz model is more sensitive to error in cone opening angle associated with pyramidal tips than to error in the sphere radius associated with spherical tips.

Although the positive correlation between cell and substrate moduli was observed with the pyramidal tip regardless of the number of points tested, the average local cell moduli only converged after 2×2 measurements. This implies that the modulus value estimated from the average of no more than nine (and possibly as few as five) evenly selected local measurements could be equivalent to that from the average of 64 local measurements, but with significant savings in experimental time.

The parametric FEA and validation experiment results suggest that cell height and substrate stiffness result in no more than ~12% error in cell elastic modulus estimated with a spherical tip and the Hertz model. This error in elastic modulus is much less than the differences we observed between stiffnesses, and therefore would not mask trends and relative comparisons. However, because the measurement accuracy is non-linearly dependent on cell height and substrate stiffness for physiologically-relevant ranges (E_{gel}/E_{cell}~0.5–10), care must be taken when reporting absolute moduli. The sensitivity analysis presented here provides guidelines for interpretation of AFM measurement of cells on gels and should be broadly useful to the cell mechanics community.

The Young's moduli we measured locally for VICs are consistent with other studies that used AFM to show that adherent cells such as fibroblasts exhibit increasing elastic moduli from 1 to 10 kPa when grown on compliant substrates of increasing stiffness (Azeloglu et al., 2008; Solon et al., 2007). Merryman et al. (2007) reported elastic moduli of ~55 kPa for VICs grown on glass, supporting the influence of substrate stiffness on cell stiffness. The Young's moduli that we measured on VICs locally were larger than those measured using micropipette aspiration (Merryman et al., 2009; Wyss et al., 2012; Zhao et al., 2009), as anticipated based on studies with other cell types (Guilak et al., 1999; Mathur et al., 2001; Na et al., 2004; Sato et al., 1990). The correlation between α-SMA levels and VIC elastic moduli is consistent with what we (Wyss et al., 2012) and others (Merryman et al., 2007) found in suspended VICs, but this is the first demonstration that this relationship also correlates with substrate stiffness, lending physiological relevance to the findings. Further, because we measured the VICs while adherent to substrates with stiffnesses similar to that of valve tissue, the Young's moduli reported here should be more physiologically-relevant and better suited as input data for computational models than measurements made on glass or in suspension. The relationships between substrate stiffness, VIC elastic modulus, and VIC cytoskeletal organization may have implications for how hemodynamic forces regulate VIC biology and valve (patho)biology (Balachandran et al., 2011; Rajamannan et al., 2011).

Conflict of interest statement

None of the authors reports a conflict of interest.

Acknowledgments

This work was supported by the Natural Science and Engineering Research Council of Canada and the Canada Research Chairs in Mechanobiology (CAS) and Micro- and Nano-Engineering Systems (YS).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jbiomech.2013.05.001.

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