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Extracellular matrix presentation modulates vascular smooth muscle cell mechanotransduction

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ABSTRACT

The development of atherosclerosis involves phenotypic changes among vascular smooth muscle cells (VSMCs) that correlate with stiffening and remodeling of the extracellular matrix (ECM). VSMCs are highly sensitive to the composition and mechanical state of the surrounding ECM, and ECM remodeling during atherosclerosis likely contributes to pathology. We hypothesized that ECM mechanics and biochemistry are interdependent in their regulation of VSMC behavior and investigated the effect of ligand presentation on certain stiffness-mediated processes. Our findings demonstrate that substrate stiffening is not a unidirectional stimulus—instead, the influence of mechanics on cell behavior is highly conditioned on ligand biochemistry. This "stiffness-by-ligand" effect was evident for VSMC adhesion, spreading, cytoskeletal polymerization, and focal adhesion assembly, where VSMCs cultured on fibronectin (Fn)-modified substrates showed an augmented response to increasing stiffness, whereas cells on laminin (Ln) substrates showed a dampened response. By contrast, cells on Fn substrates showed a decrease in myosin light chain (MLC) phosphorylation and elongation with increasing stiffness, whereas Ln supported an increase in MLC phosphorylation and no change in cell shape with increasing stiffness. Taken together, these findings show that identical cell populations exhibit opposing responses to substrate stiffening depending on ECM presentation. Our results also suggest that the shift in VSMC phenotype in a developing atherosclerotic lesion is jointly regulated by stromal mechanics and biochemistry. This study highlights the complex influence of the blood vessel wall microenvironment on VSMC phenotype and provides insight into how cells may integrate ECM biochemistry and mechanics during normal and pathological tissue function.

1. Introduction

Cells have evolved sophisticated systems to sense and respond to extracellular cues. In complex multicellular organisms, most cells are embedded within an extracellular matrix (ECM) composed of proteins and polysaccharides organized into a supramolecular structure. Considerable research effort has focused on the regulatory role of ECM biochemistry and, more recently, mechanics, on cellular function (Brown et al., 2010; Byfield et al., 2009; Calve and Simon, 2012). The mechanical properties of the ECM play a critical role in regulating signaling pathways that underlie cell adhesion, migration, proliferation, and differentiation (Chen et al., 2004; Chettimada et al., 2012), but mechanotransduction is most commonly studied by culturing cells on deformable gels of defined stiffness coated with a specific ECM cell adhesion protein (Brown et al., 2010; Calve and Simon, 2012; Cipolla et al., 2002). While many cells show stiffness-dependent responses, recent studies suggest that ECM ligand biochemistry and stiffness function together to modify cell behavior (Discher, 2005; Geiger et al., 2009). For example, skeletal muscle cells show enhanced cell plasticity and migration on provisional matrices containing tenascin C and Fn of reduced stiffness, while stiffer ECM containing laminin (Ln) suppresses migration (Engvall and Ruoslahti, 1977). Another study showed that M2 melanoma cells null in filament A do not show stiffness-dependent changes in cell spreading on gels coated with type I collagen (Col) but do show increased spread area with stiffness on Fn-coated gels (Discher, 2005). Despite these observations, the interaction of ECM biochemistry and

Abbreviations: ECM, extracellular matrix; VSMC, vascular smooth muscle cell; Col, collagen; Fn, fibronectin; Ln, laminin; kPa, kilopascal; PAAM, polyacrylamide; AFM, atomic force microscopy; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco's modified Eagle medium; EDTA, ethylenediaminetetraacetic acid; TBS, Tris-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; pMLC, phospho-myosin light chain; TFM, traction force microscopy; TF, traction force.

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mechanics remains poorly understood in the context of cellular physiology.

Changes in the biochemical and mechanical properties of the ECM are correlated with many complex diseases including cancer and atherosclerosis. In the latter, the local elastic modulus of the blood vessel wall increases from approximately 40 ± 12 kPa to 110 ± 70 kPa as the disease progresses from initial injury to advanced plaque (Guo et al., 2012). Atherosclerotic and stenotic regions also exhibit significant changes in ECM composition and structure. While the precise profile of these changes is dependent on disease phenotype, multiple studies have documented a relative increase in fibronectin and certain types of collagen, and a decrease in laminin and elastin at sites of atherosclerotic injury (Hachani et al., 2011; Hedin et al., 1988; Hong et al., 2011).

In the present study, we sought to understand how ECM biochemistry may modulate the effect of substrate stiffening on vascular smooth muscle cells (VSMCs). VSMCs are intimately involved in atherosclerosis and restenosis, exhibiting increased migration, proliferation, and ECM remodeling, leading to neointimal expansion and plaque growth (Isenberg et al., 2009; Lacolley et al., 2012; Liu et al., 2010). Previous studies have shown that VSMC phenotype is regulated by ECM mechanics (Long et al., 2008; Mammoto and Ingber, 2010) and biochemistry (Matsumoto et al., 2002; McDaniel et al., 2007; Munevar et al., 2001), but little is known about the combined effects of these variables in the context of VSMC pathology.

To this end, we investigated diverse processes in VSMCs as a function of stiffness conditioned on the presence of Col, Fn, or Ln as the adhesive ligand. Our results demonstrate that the effect of increasing stiffness on VSMC phenotype is critically subject to ligand presentation. Whereas VSMCs showed increased attachment and spreading with increased stiffness on gels containing Col or Fn, the opposite response was observed on Ln-coated gels. Conversely, myosin light chain phosphorylation was augmented by increasing stiffness on Ln but reduced on Fn. When the combined influence of Fn and Ln on VSMC attachment was evaluated on substrates functionalized with varying ratios of the two ECM proteins, we observed that neither protein was dominant in influencing VSMC response to substrate stiffness. Instead, VSMCs appear to process diverse stimuli from the ECM by integrating multiple signals, rather than preferentially responding to one over another.

Collectively, these results make a novel and important contribution to our understanding of mechanotransduction in differentiated cell lineages: within a single cell type, the effects of substrate stiffness are not uniform and are critically dependent on ligand biochemistry. This finding has significant implications for understanding the role of extracellular mechanics in the perpetuation of specific cellular states under healthy and pathologic conditions. More specifically, our study sheds new light on the regulation of VSMC phenotype *in vivo*, highlighting the critical role of ECM composition in mediating the influence of mechanical changes within the blood vessel wall.

2. Results

We studied the degree to which stiffness effects are conditioned on ligand presentation (termed "stiffness-by-ligand effects") in VSMCs by culturing cells on 25 kPa and 135 kPa substrates functionalized with equal surface densities (by mass) of Col, Ln, or Fn. To determine how VSMC spreading is affected by these variables, we quantified cell area using fluorescent image analysis and averaged these data for each condition (Fig. 1A). On Fn and Col substrates, cells exhibited a higher degree of spreading on stiffer (135 kPa) substrates (1.2 and 1.3-fold, respectively, $p < 0.05$), whereas on Ln, spreading was higher on softer (25 kPa) substrates (1.2-fold, $p < 0.05$).

To determine whether ECM presentation also modulates the effect of stiffness on VSMC proliferation (Fig. 1B), we calculated the percent of proliferating cells per substrate by dividing the number of nuclei stained with EdU (an indicator of DNA replication) by total DAPI stained nuclei. Increasing stiffness affected VSMC behavior on Col substrates only, where proliferation showed a modest increase on stiffer substrates (1.3-fold increase, $p < 0.05$).

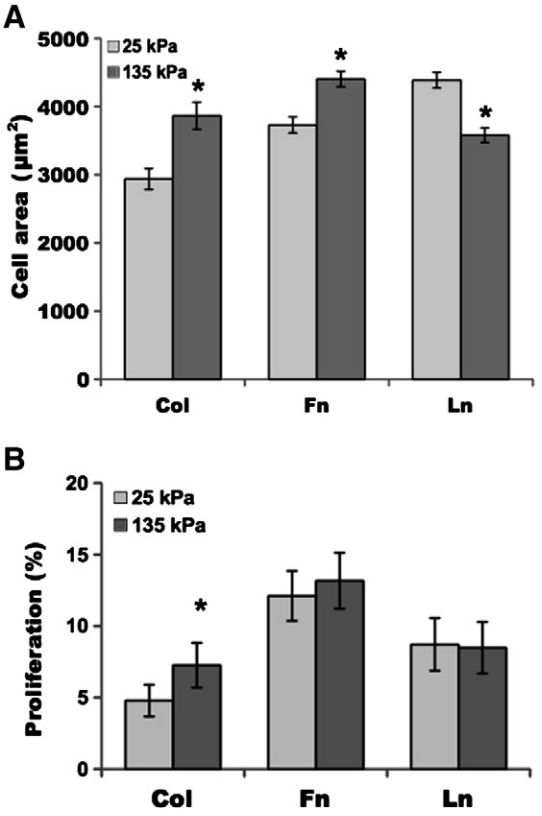

Fig. 1. Stiffness-by-ligand regulation of VSMC spreading and proliferation. Cell proliferation and spreading were quantified with fluorescent microscopy and image analysis. ECM ligand type modulates stiffness-induced effects on VSMC spreading (A), with spreading increasing on Fn and Col but decreasing on Ln as stiffness increases from 25 to 135 kPa. Proliferation (B) does not show substantial changes with either stiffness or ligand presentation. For A, data are the product of 4–6 biological replicates ($n = 200$–500 cells per condition), and data from all experiments were pooled by stiffness and ligand. For B, data are the product of 5–7 biological replicates ($n = 10$–14 gels). Asterisks (*) indicate statistically significant differences from 25 kPa as determined with a two-tailed (A) or paired one-tailed (B) *t*-test. For this and all figures, error bars represent standard error and $p < 0.05$ indicates statistical significance.

We then assessed whether cell shape and actin polymerization are also subject to stiffness-by-ligand effects on Fn versus Ln substrates. Cell aspect ratio, a measure of cellular elongation, decreased with increasing stiffness on Fn (0.8-fold, $p < 0.05$) but not Ln substrates (Fig. 2A–C). Examining the aspect ratio distribution (represented by a probability density function) across cell populations revealed significant overlap between conditions, with the difference in the means on Fn being due to 135 kPa substrates supporting fewer highly elongated cells (aspect ratio >6.0) than 25 kPa substrates. We also measured f-actin polymerization in individual cells (controlling for cell area) (Fig. 2D-F) and found more f-actin per cell on stiffer Fn substrates (1.3-fold, $p < 0.05$), but less f-actin per cell on stiffer Ln substrates (0.6-fold, $p < 0.05$). From the distributions of f-actin across conditions, we observed that these effects were due to small but persistent increases in the number of cells exhibiting higher levels of f-actin polymerization on 135 kPa Fn and 25 kPa Ln substrates, respectively. By contrast, a qualitative assessment of actin distribution within VSMCs on each substrate revealed no significant changes in cytoskeletal morphology across the four conditions (data not shown).

We also investigated stiffness-by-ligand effects on VSMC attachment. The stiffer Fn and Col substrates showed increased cell attachment compared to their respective softer substrates (2.6- and 1.3-fold increase, respectively, $p < 0.05$) (Fig. 3A). By contrast, cell attachment decreased to 0.69-fold ($p < 0.05$) on the stiffer versus softer Ln substrates.