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Extracellular matrix type modulates cell migration on mechanical gradients

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ABSTRACT

Extracellular matrix composition and stiffness are known to be critical determinants of cell behavior, modulating processes including differentiation, traction generation, and migration. Recent studies have demonstrated that the ECM composition can modulate how cells migrate in response to gradients in environmental stiffness, altering a cell's ability to undergo durotaxis. These observations were limited to single varieties of extracellular matrix, but typically cells are exposed to environments containing complex mixtures of extracellular matrix proteins. Here, we investigate migration of NIH 3T3 fibroblasts on mechanical gradients coated with one or more type of extracellular matrix protein. Our results show that NIH 3T3 fibroblasts exhibit durotaxis on fibronectin-coated mechanical gradients but not on those coated with laminin, demonstrating that extracellular matrix type can act as a regulator of cell response to mechanical gradients. Interestingly, NIH 3T3 fibroblasts were also observed to migrate randomly on gradients coated with a mixture of both fibronectin and laminin, suggesting that there may be a complex interplay in the cellular response to mechanical gradients in the presence of multiple extracellular matrix signals. These findings indicate that specific composition of available adhesion ligands is a critical determinant of a cell's migratory response to mechanical gradients.

1. Introduction

Extracellular matrix stiffness has increasingly come to be seen as an important regulator of cell behavior, driven by observations of numerous cell functions modulated by substrate stiffness and by observations of the prevalence of tissue stiffness changes in disease. Physiological stiffness is known to vary over many orders of magnitude, from as low as tens to hundreds of pascals in brain and adipose tissue to megapascals and gigapascals in tendon and in bone, respectively, with the majority of soft tissues falling in the stiffness range of hundreds of pascals to tens of kilopascals [1,2]. Additionally, matrix stiffness is known to change in both development and disease, likely leading to changes in cell behavior. For instance, in pulmonary fibrosis, increasing stiffness of lung tissue is believed to contribute to increased myofibroblast proliferation [3–5]. Given the importance of stiffness in these processes, a substantial effort has been made to understand how changes in stiffness modify cell behavior in vitro. Stiffness has been demonstrated to alter cell adhesion and proliferation rates [6], cell-cell attachment [7], sensitivity of cells to soluble factors [8,9], differentiation of cells into various lineages [10–12], magnitude of traction forces applied to substrates [13–15], and rates of cell migration [16,17]. In addition to responding to changes in the absolute stiffness of their environment, cells have been observed to respond to gradients in environmental stiffness. Durotaxis, a process by which cells migrate preferentially from regions of lower to higher substrate stiffness, has been documented in a variety of cell types on mechanical gradients spanning a wide range of absolute and relative substrate stiffnesses [18,19].

Differences in the stiffness of tissue matrices are accompanied by differences in extracellular matrix composition, and the type of extracellular matrix in a given tissue will also function as a major determinant of the behavior of cells in that tissue [20]. The interplay between mechanical stiffness and matrix composition in normal and pathological physiology is only now becoming appreciated. Recent studies in which tissue stiffness was mapped by AFM indentation have identified heterogeneities that indicate the presence of mechanical stiffness gradients in both healthy and diseased tissues. These measurements indicate the presence of a wide range of absolute stiffnesses and gradient strengths in vivo [21–26]. Importantly, such stiffness gradients have been demonstrated to accompany changes in extracellular matrix composition in a number of diseases. For instance, in pulmonary fibrosis, local increases in lung parenchymal tissue stiffness are accompanied by an increase in collagen I concentration [4], and in breast cancer an increase in stiffness from the tumor core to the periphery is associated with increased levels of collagen I and laminin [24]. In atherosclerosis, a disease characterized by the thickening of the intimal region of the arterial wall, changes in the mechanics and composition of the intimal matrix occur in conjunction with accumulation of smooth

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muscle and inflammatory cells [27–29]. Stiffness mapping experiments have shown that plaque stiffness is spatially heterogeneous, and that these changes can be histologically related to extracellular matrix composition of the plaque [26,30]. Given the increasing number of examples for which changes in extracellular matrix composition in disease are coupled to changes in mechanical properties of diseased tissue, there is a need for in vitro studies that systematically explore how the cellular response to stiffness is altered by extracellular matrix composition.

Extracellular matrix composition has been demonstrated to modulate in vitro responses to substrate stiffness in behaviors such as cell adhesion, spreading, differentiation, junction formation, traction force generation, and matrix production [31–36]. These studies suggest that many observed responses to changes in stiffness will be subject to the type of extracellular matrix available for cells to adhere to. Thus, it will be important to assess whether migratory responses of cells to mechanical gradients are also regulated by extracellular matrix composition. We recently reported an experimental system to generate polyacrylamide gels with highly reproducible linear mechanical gradients in substrate stiffness and used it to explore whether migration of vascular smooth muscle cells on mechanical gradients was extracellular matrix type-dependent [37]. However, the effect of combinations of extracellular matrix on the cellular response to mechanical gradients has yet to be explored. To address this, we have utilized mechanical gradient hydrogels coated with different extracellular matrix types to study migration of NIH 3T3 fibroblasts. Cells were cultured on mechanical gradient hydrogels with an 18.6 kPa/mm gradient between 1 kPa and 25 kPa low and high stiffness regions coated with fibronectin, laminin, and a 50:50 ratio of fibronectin and laminin by mass. We observed durotaxis behavior on fibronectin, as has been previously reported, and observed random migration on laminin and mixed-matrix gradients. Our results illustrate that matrix-type may act as a regulator of a cell's ability to respond to gradients in environmental mechanics, and the lack of observable durotaxis on mixed-matrix gradients suggests that the presence of laminin could act to inhibit the durotactic response usually seen in response to fibronectin-coated gradients.

2. Materials and methods

2.1. Gradient gel fabrication and surface functionalization

Polyacrylamide gels featuring gradients in mechanical compliance between uniform stiffness control regions were prepared as previously described [37]. Briefly, gradient generator slides were prepared by micropatterning a hydrophobic silane boundary around an adhesive, hydrophilic silane region of defined geometry using maskless lithography [38]. The micropattern features a “dumbbell-shaped” geometry, with large reservoir regions for low- and high-stiffness polyacrylamide gel solutions connected by a narrow gradient mixing region. A patterned slide and a bare, sacrificial glass slide were sandwiched around a pair of 250 μm teflon spacers to form a gradient generator device into which polyacrylamide gel solutions could be injected for controlled mixing. Low- and high-stiffness pre-gel solutions were prepared with 10% acrylamide monomer (Biorad), 0.1% (low stiffness) or 0.5% (high stiffness) N,N′-methylenebisacrylamide (Biorad), amine-reactive cross-linker NHS-ester acrylic acid (Sigma), and I2959 photoinitiator (Irgacure) in PBS adjusted to pH 6.0 with hydrochloric acid. The two pre-gel solutions were injected into either side the gradient generator device, meeting in the center of the gradient mixing region. The pre-gel solutions were allowed to diffuse across the mixing region for 10 min in order to establish a linear gradient in bis-acrylamide cross-linker concentration, at which point the devices were irradiated with ultraviolet light for 240 s to initiated polymerization of the gel solutions. Sacrificial slides were removed, and the resulting polyacrylamide gradient gels were stored in PBS adjusted to pH 6.0. The resulting gradient gels featured 1 mm gradient regions with an 18.6 kPa/mm gradient between 1 and 25 kPa low- and high-stiffness control regions [37].

Polyacrylamide gradient gels were functionalized with extracellular matrix proteins by incubation in concentrated ECM solutions. Covalent attachment to the gel surface was facilitated by reaction with NHS-ester groups incorporated into the polyacrylamide backbone by inclusion of NHS-ester acrylic acid in the pre-gel solutions. Polyacrylamide gels were incubated in a solution of fibronectin (Millipore), laminin-1 (Sigma), or a 50:50 mixture by mass of fibronectin and laminin at 5 μg/cm^2 surface concentration in PBS adjusted to pH 8.0 for two hours at room temperature. After incubation, gels were rinsed three times with PBS then stored overnight at 4 °C in 1 M glycine in PBS to ensure there would be no unreacted NHS-ester groups remaining. Gels were rinsed three times with PBS then stored in PBS prior to use.

2.2. Cell culture and reagents

NIH/3T3 fibroblasts (ATCC) were cultured in high glucose Dulbecco's modified Eagle's medium (Gibco) supplemented with L-glutamine (Gibco) and 10% bovine calf serum (Hyclone). Cells were cultured on plasma-treated tissue culture dishes (Corning) and passaged with 0.05% trypsin-EDTA (Gibco).

2.3. Cell migration on gradient gels

NIH/3T3 fibroblasts were seeded on gradient gels at a density of 1500/cm^2 and were allowed to attach for 3 h prior to imaging. 3 or more gels were used for each experimental condition in independent trials. For soluble laminin treatment experiments, cells were suspended at 10^6/mL concentration in culture media and incubated with 50 μg/mL laminin for ten minutes prior to seeding. Cells adhered to the low stiffness, high stiffness, and gradient stiffness regions of each gel were imaged in 20 min intervals over a period of 18 h using an inverted optical microscope (Zeiss) equipped with a motorized stage (Ludl Electronic Products) and a custom-built incubator to control temperature, CO_2, and humidity.

2.4. Quantification of cell migration parameters

Cell centroid positions were recorded in ImageJ at each time point. Migration tracks were assembled as vector displacements between the centroid positions recorded from sequential slices and were analyzed using a custom R script. For each cell tracked, X-displacement was calculated as the vector component of displacement in the direction of increasing substrate stiffness, and the durotactic index was calculated as the X-displacement divided by the cumulative path length. Statistical analysis of cell migration data was performed using one-factor or two-factor ANOVA as needed and Tukey's honest significant difference test for post-hoc analysis where appropriate.

3. Results and discussions

We sought to investigate whether previously observed responses of NIH 3T3 fibroblasts to gradients in mechanical stiffness were dependent on extracellular matrix type and how cells migrated on mechanical gradients in the presence of multiple matrix molecules. We previously observed that bovine vascular smooth muscle cells would undergo durotaxis on gradient gels coated with fibronectin, but would migrate randomly on laminin [37]. Here, we asked whether the same behavior could be observed in another cell type previously reported to exhibit durotaxis, and investigated effects of a mixture of both fibronectin and laminin on the migratory response to mechanical gradients. The effect of extracellular matrix composition on migration of NIH 3T3 fibroblasts on mechanical gradient gels and uniform stiffness control gels was assessed qualitatively by plotting the trajectory of each cell tracked relative to a common origin. A random subset of cell tracks from each experimental condition is shown in Fig. 1. Visual inspection of cell