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Augmentation of integrin-mediated mechanotransduction by hyaluronic acid

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ABSTRACT

Changes in tissue and organ stiffness occur during development and are frequently symptoms of disease. Many cell types respond to the stiffness of substrates and neighboring cells in vitro and most cell types increase adherent area on stiffer substrates that are coated with ligands for integrins or cadherins. In vivo cells engage their extracellular matrix (ECM) by multiple mechanosensitive adhesion complexes and other surface receptors that potentially modify the mechanical signals transduced at the cell/ECM interface. Here we show that hyaluronic acid (also called hyaluronan or HA), a soft polymeric glycosaminoglycan matrix component prominent in embryonic tissue and upregulated during multiple pathologic states, augments or overrides mechanical signaling by some classes of integrins to produce a cellular phenotype otherwise observed only on very rigid substrates. The spread morphology of cells on soft HA-fibronectin coated substrates, characterized by formation of large actin bundles resembling stress fibers and large focal adhesions resembles that of cells on rigid substrates, but is activated by different signals and does not require or cause activation of the transcriptional regulator YAP. The fact that HA production is tightly regulated during development and injury and frequently upregulated in cancers characterized by uncontrolled growth and cell movement suggests that the interaction of signaling between HA receptors and specific integrins might be an important element in mechanical control of development and homeostasis.

1. Introduction

Changes in tissue and organ stiffness are frequently symptoms of diseases such as cancer [1], liver fibrosis [2], and atherosclerosis [3], and these physical changes have been suggested to contribute to and not only be symptoms of the disease. For example, liver stiffness, as quantified by its shear modulus, increases during experimentally-triggered liver fibrosis prior to increased matrix deposition or altered cell morphology [4] by a mechanism involving lysyl oxidase [5]. Similarly, the development of atherosclerotic lesions in an apoE null mouse model can be reversed by inhibition of abnormal lysyl oxidase activity and subsequent reversal of arterial stiffening [3]. Such results suggest that changes in tissue mechanics that can activate hepatic stellate cells [6], portal fibroblasts [7] or vascular smooth muscle cells [8] in the affected organs precede and therefore might cause or at least contribute to development of the pathologic state. The response of cells to abnormal matrix stiffness can also render them resistant to chemotherapeutic agents, possibly because of the changes in the cytoskeleton-membrane

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interface [9]. Such effects in vivo have motivated studies in vitro to determine how physical properties such as increased cellular tension or adherence to substrates of differing stiffness affect cell function under conditions where physical stimuli can be isolated from biochemical signals.

Many cell types alter their structure and function in vitro depending on the mechanical properties of the materials to which they adhere [10] and on the type of adhesion receptor by which they bind [11–13]. Most studies of cellular mechanosensing have used inert, non-adhesive, soft materials for which mechanical properties can be controlled, and coupled these substrates to cell adhesion proteins or synthetic ligands that engage specific transmembrane proteins. Independent control of mechanical and adhesive changes in the substrates has been essential to demonstrate that changes in substrate viscoelasticity *per se*, and not a coincident change in cell signaling caused by altered adhesion protein presentation causes the change in phenotype. The large majority of mechanosensing studies have used the integrin ligands fibronectin, collagen, laminin, or RGD-containing peptides as the adhesive anchor, and often polyacrylamide or other hydrogels such as alginate, poly(ethylene glycol) or methacrylated hyaluronan to produce substrates softer than 50 kPa. A smaller but growing number of studies have investigated mechanosensing mediated by cadherins to mimic cell–cell junctions [13,14].

Studies in vitro of cells anchored to substrates through integrins or in some cases cadherins, show that a common, though not universal, response of cells to substrate stiffness is an increase in adherent area, increased traction forces applied to the substrate, assembly of large actin bundles called stress fibers, and activation of signaling intermediates such as small GTPases and tyrosine kinase pathways that regulate actin assembly and acto-myosin contractility [15,16]. The inference from such studies is that most cell types actively probe the mechanics of their environment by acto-myosin dependent forces, which increase when the resistance imposed by the substrate increases, and the feedback between cell and substrate reorganizes the cytoskeleton to achieve a homeostatic state appropriate for each physical context [17]. Substrate stiffness and the resulting increase in cell-generated forces can also increase activity of matrix-bound growth factors such as TGF-beta, that further increase development of the phenotype associated with growth of stiff substrates [18].

Response to substrate stiffness is highly cell-type specific, and neurons for example, have a unique response to stiffness, in which matrix stiffness greater than that of the normal CNS tissue inhibits neurite outgrowth and growth cone spreading [19–21]. Myocytes have a particularly striking and well-documented response to matrix stiffness, with a distinct optimum for development of sarcomeres and an elongated shape that depends on both matrix stiffness [22–24] and the type of adhesion receptor [13,14,25]. On polyacrylamide (PAA) gels that are laminated with ligands for integrins, cardiac myocytes develop well organized sarcomeres only when cultured on substrates with elastic moduli in the range of 10 kPa–30 kPa, near those of the healthy tissue. On stiffer substrates (>60 kPa) approximating the damaged heart, myocytes form stress fiber-like filament bundles but lack organized sarcomeres or an elongated shape. On soft (<1 kPa) PAA gels myocytes exhibit disorganized actin networks and sarcomeres. On N-cadherin-coated PAA gels, the response is similar but the optimum is shifted to slightly lower stiffness (5 kPa) [14].

In contrast to the simplified chemical composition of soft substrates used for mechanosensing studies in vitro, cells engage their extracellular matrix (ECM) in vivo both by mechanosensitive adhesion complexes and by other surface receptors for ECM components that cannot act as adhesive anchors, but that potentially modify the mechanical signals transduced at the cell/ECM interface. Such ECM components include not only growth factors such as TGF-beta but also proteoglycans and glycosaminoglycans such as hyaluronic acid that constitute a major fraction of the total ECM content, and that change in abundance during development, wound healing, and disease. For example, during development, cardiac myocytes assemble and organize their internal structures within a complex mechanical tissue environment bounded by an especially soft (E ~ 20–100 Pa) [26,27] hyaluronan-and fibronectin-containing cardiac jelly and a considerably stiffer (E ~ 10 kPa) [23] compacted myocardial tissue. How a sarcomere forms in such a soft matrix in vivo, whereas a substrate with the same low elastic modulus prevents sarcomere formation in vitro is not known, but the transient expression of hyaluronic acid during conditions where cells mature within a very soft matrix suggests that it might contribute to the development of cell morphology in a manner that is not fully reproduced by integrin signaling alone.

Hyaluronic acid (HA) is a high molecular weight (6–7000 kDa), linear polysaccharide found in soft tissue and synovial fluid that consists of N-acetyl-D-glucosamine and D-glucuronic acid residues that give the molecule a highly negative charge. HA interacts with cells through its receptors CD44 [28], RHAMM [29], layilin [30] and ICAM-1 [31]. HA can also bind fibronectin (Fn) [32] and collagen VI [33] in vitro, suggesting that HA might modify cell adhesion to these integrin ligands. HA is synthesized by many cell types and either retained on the cell surface as a pericellular coat or cleaved from the cell and released into the extracellular matrix (ECM) [34]. HA and HA receptor syntheses are tightly regulated during development [35] and often activated during normal wound healing, especially during fetal wound repair that enables healing without scarring [36]. HA in either soluble or crosslinked forms is a commonly used simple and semi-synthetic soft material with numerous current clinical applications [37], although usually in a form that is highly modified by methacrylation or other covalent linkages that might affect its binding to HA receptors. The studies in this report test the hypothesis that the presence of long unmodified hyaluronan polymers within a matrix that also contains integrin ligands such as fibronectin alters the mechanosensing signals mediated by the activated integrin to elicit a phenotype that cannot be attained under the same mechanical conditions by integrin engagement alone.

2. Materials and methods

2.1. Cell line culture and/or isolation

Neonatal ventricular rat myocytes (NVRM) were harvested from the hearts of 1- to 3-day-old euthanized Sprague–Dawley rat pups using a cell isolation kit (Cellutron Life Technology, Baltimore, MD) as described previously [14]. Isolated cardiac myocytes were pre-plated for 1–2 h to purify the myocyte population. The cells were cultured at a density of 7000 cells/cm^2 in high serum (10% fetal bovine serum) medium (Cellutron) on the various gel substrates for 24 h. The medium was changed to low serum (2% fetal bovine serum) and maintained for another 24 h. This time period proved sufficient to allow the cells to attach and spread completely after the isolation procedure. Human mesenchymal stem cells (hMSCs) (Lonza), 3T3 fibroblasts and human umbilical vein endothelial cells (HUVECs) were cultured in their respective medium for a period of 24 h or greater.

2.2. Hydrogel substrate preparation

Polyacrylamide (PAA) and hyaluronan gels of desired stiffness were made using methods described elsewhere [10,12,14]. Briefly, the acrylamide solutions (Bio-Rad Laboratories, Hercules, CA) are polymerized using TEMED (Fisher BioReagents, Fairlawn NJ) and 10% ammonium persulfate (Fisher BioReagents). The solution was deposited on a 20-mm square glass coverslip pretreated with 3-aminopropyltrimethoxysilane (Sigma–Aldrich, St. Louis, MO) and 0.5% glutaraldehyde (Sigma–Aldrich). The gels were coated with 0.1 mg/ml of bovine fibronectin (Fn) (Sigma–Aldrich) through the cross-linker N-sulfosuccinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate (0.5 mg/ml in 50 mM HEPES buffer pH 8) (Thermo Fisher Scientific, Waltham, MA).