CELLULAR RESPONSES TO LIGAND DENSITY AND SUBSTRATE STIFFNESS

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ABSTRACT

Substrate stiffness is emerging as an important physical factor in the response of many cell types. Like other anchorage dependent cells, smooth muscle cells derived from rat aorta (A7R5 line) are found to spread more and organize their cytoskeleton and focal adhesions much more so on rigid glass and 'stiff' substrates than on 'soft' gels. Collagen density also factors into cell on gel responses, with minimal spreading on very low collagen and a weak maximum in cell spreading on intermediate collagen densities. The modest bell-shaped curves are modeled by a relatively simple expression that highlights the coupling between the ligand density and substrate stiffness. Most surprising, however, spreading on soft gels is found to be almost independent of adhesive ligand density: the minimal spreading of cells cannot be over-ridden even at high collagen densities. On soft gels, however, a fraction of GFP-actin expressing cells – but not GFP-paxillin cells – do spread. Such cells invariably show an organized cytoskeleton of stress fibers, which suggests that the cytoskeleton can uniquely override spreading limits typically dictated by soft gels. Based on these results in addition to previous work from others, we hypothesize a central role for cytoskeletal-membrane interactions in the spreading response.

INTRODUCTION

Extracellular matrix (ECM) not only presents necessary adhesive ligands to anchorage-dependent cells, it also offers a number of influential mechanical properties. Key cellular processes ranging from motility to differentiation [1] have become increasingly linked to apparent 'preference' for stiffer substrates. However, it is the coupling of ECM stiffness and ligand density, and how it modifies cellular behavior (Fig. 1), that has been a recently raised question that has not been resolved [1,2]. Previous cell on gel studies have already documented the tendency for reduced spreading of non-contractile cells on soft substrates at constant collagen coatings [1,3], and since anchorage-dependent cells have been known to require a substantial area of contact, cell area is a simple, first reflection of the cellular response to these variables.

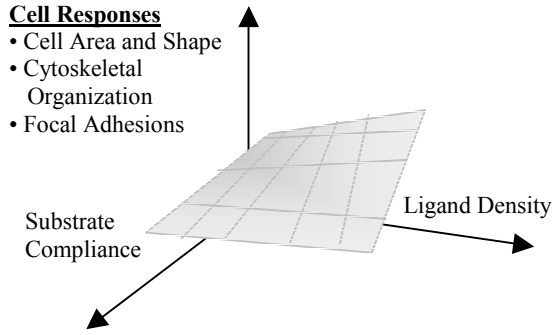

Figure 1. Cellular behavior is dictated by coupled chemical and mechanical signals [3].

Within the first 24 hours after plating, we evaluated the morphological and related structural responses of rat aorta-derived smooth muscle cells (SMC of A7R5 lineage) under the combined effects of varied collagen density and substrate compliance by examining projected cell area and other cell structural measures and perturbations. Specifically, we attached a wide range of collagen densities to polyacrylamide gels (PAG) of varied stiffnesses and ultimately found that for well-separated SMCs, the average cell area reflects a highly non-linear response. Additionly, cell shape responses are described together with assembly and perturbations of cytoskeleton and focal adhesions. The results not only indicate that SMCs are responsive in coupled ways to substrate stiffness and ligand density but also that SMCs are more clearly influenced by actin cytoskeleton perturbations balancing with substrate-dependent membrane tension.

METHODS AND MATERIALS

Experiments were performed on the A7r5 SMC line, generally known to maintain differentiation markers for α-actinin, calponin, and myosins. SMC were cultured in polystyrene flasks with Dulbeco's Modified Eagle Medium (DMEM) supplemented by 10% fetal bovine

serum. Cells were plated on PAG substrates of variable stiffness and ligand densities and observed up to 24 hours. PAG samples were prepared on glass slides with a modified method established by Wang and Pelham [4]. PAG glass supports were prepared by attaching aminosilanes to glass. N,N' methylene-bis-acrylamide (0.03% to 0.3%) was mixed into solutions of 5% acrylamide (C_3H_5NO) in distilled water, which produces a tunable elastic modulus after gelation. After the addition of polymerizing reagents, 25µl of gelling solution was added to the silanized glass surface. The solution was covered with a coverslip to create a uniform surface. Gel thicknesses were approximately 70µm, which was confirmed optically. Collagen was either adsorbed or chemically crosslinked to the gel surface by a heterobifunctional crosslinker, Sulfo-SANPAH, at 37°C overnight.

Some A7r5 cells were selectively plated and transfected in a hypo-serum environment with GFP-paxillin or GFP-actin DNA constructs for 24 hours using standard Lipofectamine transfection protocol (Invitrogen, Life Technologies). Transfected cells were introduced to the gel substrates and observed.

Gel rigidity was determined from correlated measurements of the elastic modulus determined by both nano-scale and continuum-based techniques of applied Cauchy-Lagranian stresses and strains in an established technique [5]. Nano- and continuum measurements were done as a function of crosslinker concentration, indicated a tight correlation between the two methods, and produced a working compliance range of between 1kPa (soft) and 8kPa (stiff) gel.

RESULTS

Varying the collagen density on PAG and glass was widely found to modulate cell spreading to different degrees (Fig. 2). On soft substrates, collagen density could not override the dramatically soft elasticity of the surface, while collagen density exhibited a bell-shaped response on stiffer substrates. The peaks in cell area are suggestive of biphasic cell motility versus ligand density, explained by under or over attaching the cell to ligand [6]. This effectively makes the surface non-stick or glues the cell to the substrate and ruffles the cell membrane, changing the cell shape factor linearly with ligand density. Cell-substrate detachment in spreading is less apparent which agrees with the latter phase being less prominent. Nonetheless, maximum spread area clearly shifts to lower ligand densities on softer substrates.

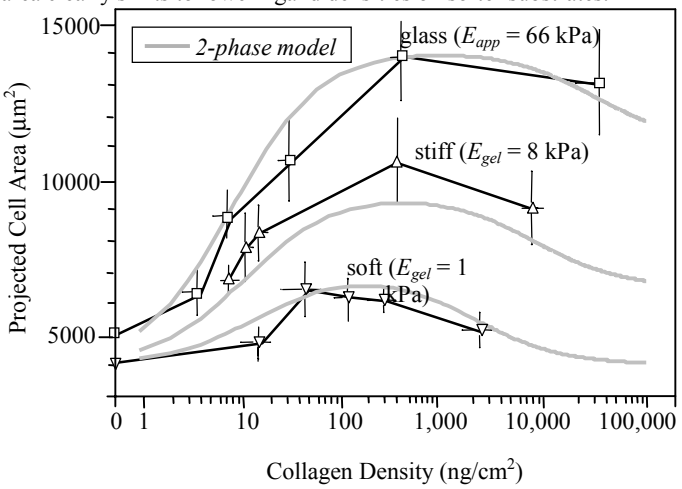

Figure 2. SMC area as a function of lingand density based on substrated elasticity.

The two potential phases of cell spreading were modeled as a single function for *Area* dependent on E or E_{app} and col = [*collagen*]. The collagen dependence has been expressed in the sum of two hyperbolic terms typical of saturable equilibrium associations. The area-promoting association of cell-substrate binding is modeled by the first term (with constant K_1) while the second term models the fractional dissociation in a separate, area-inhibiting reaction (with constant K_2). Importantly, a power law fit of *Area* (µm²) versus E (kPa) has been used to scale the area-promotion reaction obtained from cell spreading over a 24-hour time course (see Table 1). Cells cultured on glass have been designated $E = E_{eff}$ based on the maximum spreading causing an effective limit to the stiffness that a cell can remodel to match. The baseline area response of a cell in solution appears as a constant, C, and the association constants K_1 and K_2 are taken to be power laws in E_{eff} (units of kPa). The final equation (1) defines a continuous surface for the cell *Area* (µm²), yielding similar 3-dimensional information as in Fig. 1.

$$Area = C + 3000E_{eff}^{0.29}[K_1*col/(1+K_1*col)] + 3000[1/(1+K_2\,col)] \quad (1)$$

with $C = 1000$, $K_1 = 0.07*E_{eff}^{0.13}$, and $K_2 = 0.0005/E_{eff}^{0.66}$. The kinetic approach to biphasic motility were described by a ratio of dissociation rate constants of the same order of magnitude as here, and supports the notion that similar phenomena underlie the behavior in cell spreading.

Fits	4 hr parameters		24 hr parameters	
$y=ax_1^m/(K_{el}^m+x_1^m)$	$m = 0.87$	K_{el}=10kPa	$m = 1.0$	K_{el}=7.5kPa
$y=bx_1^n$	$n = 0.29$	$B = 4000$	$N = 0.37$	$B = 5500$

Table 1. Fits of SMC area versus substrate elasticity.

Transfected GFP-actin and –paxillin cells elucidated the role of substrate elasticity in cytoskeletal and adhesion expression and structure. On the softest collagen coated PAG, the cells are generally rounded with diffuse but visible GFP-actin and minimal filament and adhesion organization. Conversely, on both stiff PAG and rigid substrates pretreated with collagen, structured actin stress fibers and paxillin focal adhesions were typically observed. Again, well-spread cells predominate on collagen-coated substrates. Without collagen, both stiff gels and glass presented a small sub-population of highly spread cells with organized actin stress fibers illustrating a rule: spreading correlates well with cytoskeletal assembly. GFP-paxillin expression in these cultures, however, had little ability to overcome the dominant anti-spreading signal from untreated substrate surface.

Overall, a mechanism for cell spreading can be visualized in terms of functions of substrate chemical and mechanical properties, which in turn modulate cytoskeletal and focal adhesion structure.

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