CELL ADHESION STRENGTHENING AND FOCAL ADHESION ASSEMBLY ON MICROPATTERNED SUBSTRATES

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INTRODUCTION

Cell adhesion to extracellular matrices plays a central role in numerous physiological and pathological processes. Integrin-mediated adhesion is a highly regulated process involving receptor activation and mechanical coupling to extracellular ligands [1]. Bound integrins rapidly associate with the actin cytoskeleton and cluster together giving rise to focal adhesions, discrete complexes of structural and signaling proteins. Focal adhesions are central elements of the adhesion process, functioning as structural links between the cytoskeleton and the extracellular matrix and triggering signaling pathways that direct cell proliferation and differentiation [2]. Because the biochemical and biophysical processes in the focal adhesion complex are tightly coupled, mechanical analyses of the adhesion strength provide critical information on structure-function relationships for these specialized structures. These relationships are critical to manipulating adhesive interactions in biomedical and biotechnological applications. We have engineered surfaces with micropatterned arrays of adhesive islands that control contact area, cell shape and spreading, and focal adhesion assembly. This approach allows us to decouple the effects of cell spreading from adhesion strengthening and to analyze structure-function relationships in adhesive interactions. In this study we analyzed cell adhesion strengthening on these micropatterned surfaces.

MATERIALS AND METHODS

Surface Micropatterning

Micropatterned surfaces consisting of cell adhesive circular islands in a non-adhesive background were engineered using microcontact printing of alkanethiol self-assembled monolayers [3]. Standard photolithography techniques were used to manufacture templates of different island diameters (2, 5, 10 μm) on Si wafers. The island spacing (75 μm) was chosen to eliminate cell-cell interactions. A PDMS stamp having the desired features was then cast from the Si molds. To create micropatterns, the stamp was inked with "adhesive" (CH_3-terminated) alkanethiols and pressed onto Au-coated surfaces. The remaining area was derivatized with tri-ethylene glycol-terminated alkanethiols, which resist protein adsorption and cell adhesion. Micropatterned substrates were coated with fibronectin (FN) (10 μg/mL) for 1 hour and blocked with 1% serum albumin.

Immunofluorescence Staining for Focal Adhesion Proteins

Immunofluorescence (IF) staining was used to examine focal adhesion assembly. Cells were extracted to remove membrane and soluble cytoskeletal components, leaving behind focal adhesion structures. Extracted cells were then fixed, blocked in 5% serum, and incubated with primary antibodies against focal adhesion components followed by incubation in fluorochrome-labeled secondary antibodies.

Adhesion Strength

Cell adhesion strength was quantified using our spinning disk assay [4]. This device applies a linear range of hydrodynamic forces to adherent cells and provides sensitive measurements of adhesion strength. NIH3T3 fibroblasts were plated in the presence of 0.1% serum on FN-coated micropatterned arrays on circular coverslips for different time points, spun for 5 min, fixed, and stained with ethidium homodimer. Cell numbers at different radial positions were quantified using a motorized microscope stage and image analysis system.

Integrin Binding Analysis

Integrin binding was analyzed using a cross-linking/extraction/reversal biochemical technique [5]. Briefly, bound integrins were cross-linked to their extracellular matrix using sulfo-BSOCOES, cell and unbound receptors extracted, and the bound integrins recovered by reversing the cross-linking. Recovered integrins were separated by SDS-PAGE and transferred to PVDF membranes. Integrins were quantified by Western blotting [5].

RESULTS

Adhesion Strength on Micropatterned Surfaces

Micropatterning provided precise control over FN adhesive area, cell spreading/shape, and position and size of focal adhesions. This approach allows decoupling of cell shape/spreading from focal

adhesion formation. NIH3T3 fibroblasts seeded on patterned substrates individually adhered to the FN adhesive islands and maintained a nearly spherical shape, while the contact area conformed to the pattern dimensions. These cells remained viable and attached to the micropatterned and attached to the substrates for up to 5 days in culture for all FN-coated micropatterned island sizes. Immunofluorescence microscopy showed that focal adhesion components ($\alpha_5\beta_1$, vinculin, talin, paxillin) were localized only on the circular islands (**Fig 1**) [6]. Focal adhesions were uniformly distributed on islands smaller than 5 μm diameter while on larger islands they were more spatially segregated into discrete clusters. For all micropatterned substrates, cell adhesion strength increased rapidly at early time points and reached plateau values by 4 hours (**Fig 2**). By comparing experiments with similar available contact areas (5 μm dia islands) at different time points (15 min and 16 hr), the relative contributions of receptor clustering and focal adhesion assembly to adhesion strength were analyzed. Integrin clustering, focal adhesion formation and cytoskeletal recruitment resulted in 9-fold increases in adhesion strength (**Fig 2**) independently of cell spreading and redistribution of adhesive structures to the periphery. Furthermore, adhesion strength decreased with decreasing island diameter (**Fig 3**), indicating that focal adhesion area strongly modulates adhesion strength. In summary, these results suggest that focal adhesion size and composition regulate cell adhesion strength.

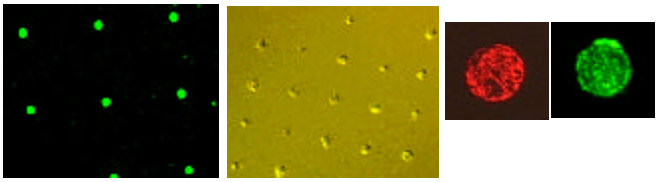

Fig 1. Micropatterned surfaces (10mm dia) to control FN adsorption (left), cell adhesion (middle), and (C) focal adhesion size ($\alpha_5\beta_1$ left, vinculin right).

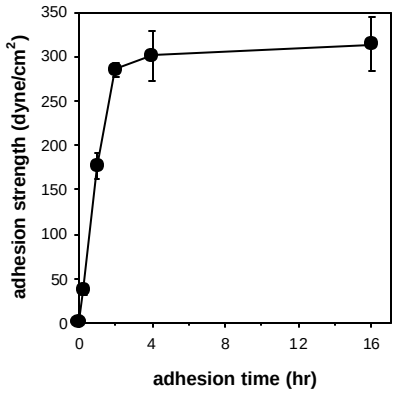

Fig 2: Adhesion strength (mean ± std. error) of NIH3T3 fibroblasts as a function of seeding time for cells seeded on 5μm diameter islands.

Bound Integrin Analysis

We are currently conducting experiments to quantify integrin binding and focal adhesion assembly to analyze the functional dependence of adhesion strength on integrin clustering and focal adhesion formation. Initial results indicate that bound receptors number increases with available contact area (**Fig 4**). It appears that the amount of integrin bound is less on the 2 μm diameter islands than on the other areas. Consistent with IF, this could be due to saturation of bound integrins in the available area on these smaller islands.

DISCUSSION

Micropatterning provides a robust method to engineer surfaces to control cell shape and spreading and focal adhesion position and size. This approach allows analysis of structure-function in adhesive interactions. Current work focuses on the role of focal adhesion position and composition and the specific contributions to adhesion strengthening.

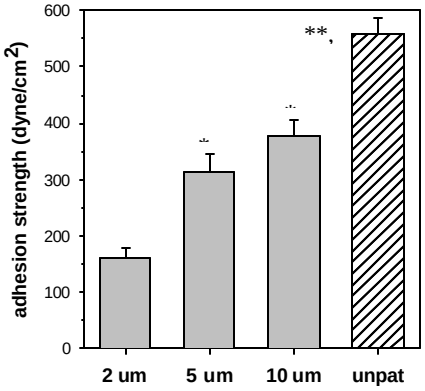

Fig 3: Steady-state (16 hr) NIH3T3 adhesion strength (mean ± std. error) as a function of adhesive island diameters showing contact-area dependence.

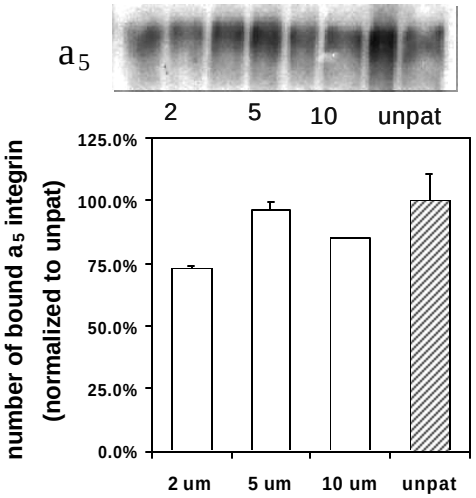

Fig 4: Bound alpha 5 integrin (mean ± std. dev.) as a function of adhesive island diameter quantified by Western blotting.

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