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Short communication

Reduced adhesive ligand density in engineered extracellular matrices induces an epithelial-mesenchymal-like transition

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ABSTRACT

A synergistic effect of biochemical and mechanical cues emanating from the extracellular matrix (ECM) on inducing malignant transformation of epithelial cells has been observed recently. However, the effect of quantitative changes in biochemical stimuli on cell phenotype, without changes in ECM component and rigidity, remains unknown. To determine this effect, we grew Madin-Darby canine kidney epithelial cells (MDCK) on gold surfaces immobilized with varying densities of cyclic arginine-glycine-aspartate (cRGD) peptide and analyzed cell morphology, cell migration, cytoskeletal organization, and protein expression. Cells grown on a surface presenting a higher density of cRGD displayed an epithelial morphology and grew in clusters, while those grown on a diluted cRGD surface transformed into an elongated, fibroblast-like form with extensive scattering. Time-lapse imaging of cell clusters grown on the concentrated cRGD surface revealed collective migration with intact cell-cell contacts accompanied by the development of cortical actin. In contrast, cells migrated individually and formed stress fibers on the substrate with sparse cRGD. These data point towards transdifferentiation of epithelial cells to mesenchymal-like cells when plated on a diluted cRGD surface. Supporting this hypothesis, immunofluorescence microscopy and western blot analysis revealed increased membrane localization and total expression of N-cadherin and vimentin in cells undergoing mesenchymal-like transition. Taken together, these results suggest a possible role of decreased biochemical stimuli from the ECM in regulating epithelial phenotype switching.

Statement of Significance

Epithelial-mesenchymal transition (EMT) is a process where adherent epithelial cells convert into individual migratory mesenchymal phenotype. It plays an important role both in physiological and pathological processes. Recent studies demonstrate that the program is not only governed by soluble factors and gene expressions, but also modulated by biochemical and mechanical cues in ECMs. In this study, we developed chemically defined surfaces presenting controlled ECM ligand densities and studied their impact on the EMT progression. Morphological and biochemical analyses of epithelial cells cultured on the surfaces indicate the cells undergo an EMT-like transition on the diluted cRGD surface while retaining epithelial characteristics on the cRGD-rich substrate, suggesting an important role of the ECM ligand density in epithelial phenotype switching.

1. Introduction

Epithelial-mesenchymal transition (EMT) is a fundamental physiological process that occurs during early embryogenesis, tissue repair, cancer, and pathology. It is characterized by the loss of the epithelial phenotype, including E-cadherin-mediated cell-cell contacts and apico-basal cell polarization, and subsequent acquisition of the mesenchymal phenotype. EMT involves profound reorganization of the actin cytoskeleton, increased migration, and eventual loss of coordinated collective motion. At the molecular level, cells undergoing EMT exhibit downregulation of E-cadherin and an increase in the expression of N-cadherin, vimentin, and fibronectin. These morphological and molecular changes are considered the hallmarks of EMT and are common to many cell types [1,2]. EMT is known to be essential for normal development

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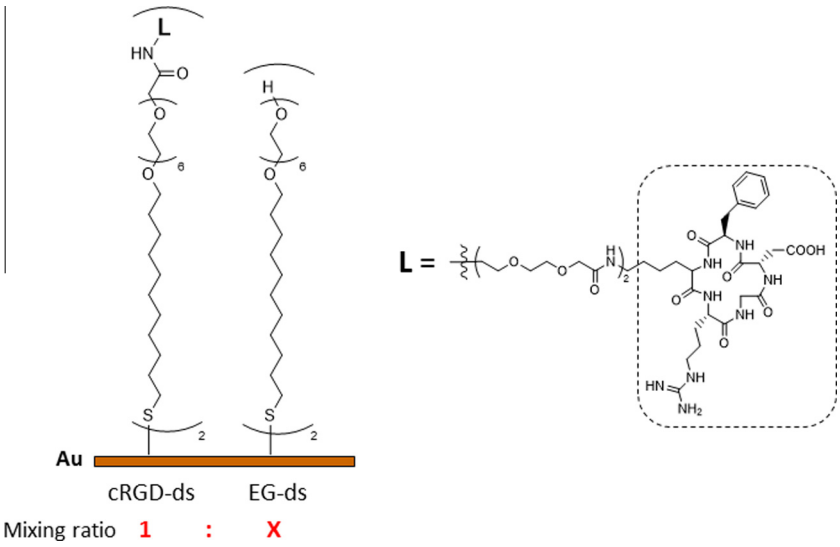
of embryo and plays a role in cancer progression. Recently, it has been demonstrated that EMT can be modulated not only by soluble factors, such as transforming growth factor beta (TGF-β) and interleukin-6 (IL-6), but also by changes in the interaction between a cell and the surrounding extracellular matrix (ECM) [3]. For example, collagen type IV has been shown to induce an EMT-like process in non-tumorigenic mammary epithelial cells by downregulating E-cadherin expression while simultaneously increasing the expression of N-cadherin and vimentin and the secretion of matrix metalloproteinase-2 (MMP-2) [4]. Similarly, during renal fibrosis, an alteration in the assembly of collagen type IV in proximal tubular epithelial cells is reported to promote TGF-β production that then induces EMT, indicating a pivotal role of ECM proteins in regulating EMT [5]. In addition, Leight et al. demonstrated the influence of matrix stiffness in deciding the fate of murine mammary gland epithelial cells and Madin-Darby canine kidney epithelial cells (MDCK) [6]. Following TGF-β stimulation, cells grown on stiff substrates underwent EMT whereas cells grown on compliant substrates underwent apoptosis via the PI3K/Akt signaling mechanism. Most such studies have focused on the impact of *qualitative* differences in the biochemical component of ECM (types and/or assembled structures of ECM proteins) and mechanical properties of ECM on EMT. Less attention is paid to the contribution of the *quantitative* difference in ECM composition on EMT induction. We have previously reported a loss in collective migration of HeLa cells, considered a hallmark of EMT, when the surface density of an ECM ligand, cyclic arginine-glycine-aspartate (cRGD), on a photoactivatable nanopatterned surface is reduced [7]. This observation deviates from our expectation that the acquisition of collective features would increase with decreasing chemical interactions between cells and the ECM. However, it points to an induction of EMT by a simple alteration in ECM ligand density as compared to qualitative changes in ECM composition that normally modify the ECM. Because HeLa cells display both epithelial and mesenchymal traits, such as constitutive expression of N-cadherin and collective migratory behavior on normal surfaces, the generalization of our previous observation needs to be tested. Therefore, we determined if variation in the density of ECM ligands could induce EMT in MDCK cells. These cells are widely used in EMT studies, as the cells inherently exhibit an epithelial phenotype but acquire mesenchymal characteristics either after transient induction by TGF-β or via stable expression of EMT-inducing genes [8–10]. Surface ECM density has been mostly controlled by changing the concentration of ECM protein solutions used for coating culture dishes. However, this strategy is not ideal for studies on EMT since molecular changes in ECM are detectable after a few days of stimulating EMT and during this experimental time course, ECM remodeling occurs through the deposition of serum-derived proteins, secreted proteins as well as by enzymatic digestion. Therefore, we prepared a chemically defined surface to correlate surface ECM ligand density to EMT (Scheme 1). cRGD peptide was used as a model ECM ligand [11]. Compared to linear RGD, cRGD is known to have high stability due to the presence of a cyclo ring that is resistant to proteolysis and has a high affinity for $\alpha_V\beta_3$-integrin receptors [12], thereby enhancing cell adhesion. Therefore, it is an ideal candidate for peptide-mediated cell adhesion studies. We conjugated cRGD ligands to hexaethylene glycol (EG_6)-terminated disulfide (cRGD-ds) and mixed them with non-cRGD-conjugated EG_6-terminated disulfide (EG_6-ds) in a given ratio. Mixing results in the formation of self-assembled monolayers (SAMs) with tuned surface cRGD densities on gold substrates. It should be noted that the EG group prevents protein adsorption to the surface of the substrate. Hence, changes in surface composition occurring due to nonspecific adsorption of serum-derived proteins are minimized over the first few days in culture. Such a surface design, therefore, enables us to investigate the effect of changes in ECM ligand density on cell phenotype within a few days of culture. The observed alterations in cellular characteristics on the reduced cRGD-functionalized surface suggest the involvement of a decrease in ECM chemical composition, at least in the early stage of EMT.

2. Materials and methods

2.1. Materials

Gold substrates were prepared by consecutively vacuum depositing a 5-nm titanium layer and a 20-nm gold layer on a glass coverslip with an E-beam evaporator. The rigidity of the substrates were expected to be in the order of gigapascals, based on literature [13], as the base material is normal glass coverslip. Antibodies and reagents were purchased as follows: mouse monoclonal anti-N-cadherin antibody (BD Transduction Laboratories™, BD Biosciences, NC, USA), rabbit polyclonal anti-E-cadherin antibody (H-108, Santa Cruz Biotechnology, Dallas, TX, USA), Alexa Fluor 546-conjugated goat anti-rabbit IgG (H+L) and Alexa Fluor 546-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (H+L) (Life Technologies, Eugene,



Scheme 1. Mixed self-assembled monolayers used in this study. An adhesive ligand (cRGD-ds) and a protein-repellent ligand (EG_6-ds) were mixed in a given ratio to form self-assembled monolayers with tuned surface densities of the adhesive ligand. The moiety surrounded by dotted line represents the cyclic [Arg-Gly-Asp-D-Phe-Lys] peptide.