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Effect of RGD secondary structure and the synergy site PHSRN on cell adhesion, spreading and specific integrin engagement

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Abstract

The relationship between the form of cell adhesion, ligand presentation, and cell receptor function was characterized using model Langmuir–Blodgett supported films, containing lipid-conjugated peptide ligands, in which isolated variables of the ligand presentation were systematically altered. First, the conformation of an adhesive Arginine–Glycine–Aspartic acid (RGD) peptide was varied by synthesizing linear and looped RGD peptide-containing amphiphiles and subsequently measuring the impact on the function of human umbilical vein endothelial cells. Secondly, the contribution of non-contiguous ligands to cellular engagement was assessed using multi-component biomimetic films. The peptide amphiphiles were composed of fibronectin-derived headgroups—GRGDSP, and its synergy site Pro–His–Ser–Arg–Asn (PHSRN)—attached to hydrocarbon tails. The peptide amphiphiles were diluted using polyethylene glycol (PEG) amphiphiles, where PEG inhibited non-specific cell adhesion. Cells adhered and spread on GRGDSP/PEG systems in a dose-dependent manner. The presentation of GRGDSP influenced integrin cell surface receptor specificity. Results demonstrated that β_1-containing integrins mediated adhesion to the linear GRGDSP presentation to a greater extent than did the $\alpha_v\beta_3$ integrin, and looped GRGDSP preferentially engaged $\alpha_v\beta_3$. GRGDSP/PHSRN/PEG mixtures that closely mimicked the RGD–PHSRN distance in fibronectin, enhanced cell spreading over their two-component analogues. This study demonstrated that controlling the microenvironment of the cell was essential for biomimetics to modulate specific binding and subsequent signaling events.

Keywords: Looped RGD; Cyclic RGD; PHSRN; HUVEC; $\alpha5\beta1$ integrin; $\alpha_v\beta_3$ integrin

1. Introduction

Control of the microenvironment of the cell provides the biomaterials engineer with the opportunity to modulate cellular events such as growth, differentiation, and angiogenesis, influence supramolecular structures in tissue development; it provides the molecular biologist a means to connect extracellular interactions with biochemical events within the cytoplasm and nucleus of the cell [1]. Consequently, a maturing theme in the biomaterials field is the design, synthesis, and modification of materials that selectively interact with cells through specific biomolecular recognition events [2]. A wide range of techniques in which the microenvironment of the cell is controlled and the biomaterial interface is rendered bioactive, are being employed to create functionalized substrates [3]. A promising approach is the biomimetic modification of the material in which peptides containing the adhesion domains of extracellular matrix proteins are attached to the interface [3,4].

One of the most commonly used ligand in biomaterials and the most physiologically ubiquitous binding motif is the short peptide sequence arginine–glycine–aspartic acid (RGD) [5]. RGD was identified through competitive binding assays involving the integrin family of cell surface receptors. Studies have shown that RGD-modified interfaces can promote integrin-mediated adhesion and migration [3,6]. Additional peptides, such as REDV [7], PHSRN [8] and KNEED [9,10] from fibronectin, YIGSR from

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laminin [11], and FHRRIKA from heparin [12], have been explored when the peptide confers a specific advantage to the substrate or serves as a new design paradigm.

Immobilization strategies have exploited RGD peptide presentation as a means to alter the bioactivity of a substrate. Crystallization of the III_7–III_{10} repeats of fibronectin showed that the RGD in the 10th III repeat is presented in a looped structure that extends 10 Å away from the face of the protein [13]; thus, additional specificity and activity may be imbedded in the peptide structure and presentation. Studies have shown that a more constrained, cyclic or looped, peptide versus a linear one exhibits a higher affinity for the $\alpha_v\beta_3$ and $\alpha_3\beta_1$ integrins [14–17]. This hypothesis was originally suggested by Pierschbacher and Ruoslahti [18]. Additionally, at neutral pH cyclic RGD is more stable than linear RGD in solution [19], and cyclization of linear peptides improves stability against enzymatic degradation [20].

Recently, multi-component peptide systems containing both RGD (the primary recognition site for $\alpha_5\beta_1$ integrins, in fibronectin III_{10} repeat) and PHSRN (the synergy site for $\alpha_5\beta_1$, in fibronectin III_9 repeat), demonstrated that a more complex biomaterial interface is capable of providing increased $\alpha_5\beta_1$-mediated adhesion and instructing the cells to adhere, spread, differentiate, migrate, and mineralize more effectively than RGD alone [21–31].

The Langmuir–Blodgett (LB) technique was used in this study to create highly structured supported membranes with multiple adhesive peptide amphiphiles. The peptide amphiphile structure includes dialkyl ester tails that allow control over their length (C_{16} or C_{18}), a glutamic acid (Glu) linker, a $-(CH_2)_2-$ spacer and a headgroup that contains the bioactive sequence. The tails serve to align the peptide strands and provide a hydrophobic surface for self-association and interaction with other hydrophobic surfaces [32]. LB films incorporating peptide amphiphiles represent a model experimental system to study ligand–receptor interactions with several advantages over other surface immobilization methods. For example, the LB technique allows for precise control of peptide surface density and orientation, mixing of multi-component systems, and the possibility of potential peptide denaturation is minimal because the functionalized surface is created by physical deposition rather than chemical reaction.

Using the LB technique, membranes supported on mica were created here, where the peptide headgroups were presented on the exterior face of the bilayer. Peptide amphiphiles that mimic the adhesion domain of the extracellular protein fibronectin, were constructed from mixtures of peptide amphiphiles and polyethylene glycol (PEG) amphiphilic molecules. PEG has been used extensively as material that is non-adhesive to biological molecules [33,34], and is utilized here to dilute the surface concentration of the peptide amphiphile. LB isotherms were used to verify that the synthesized peptide amphiphiles formed stable supported bilayers. The resulting biofunctional surfaces facilitated the study of cell adhesion phenomena. Cell adhesion, spreading, and receptor specificity experiments independently explored how human umbilical vein endothelial cells (HUVEC) responded to different presentations of multi-component systems of linear and looped GRGDSP, PHSRN, and PEG amphiphiles. Adhesion and spreading of HUVEC on supported bilayers utilized macroscopic measurements of cell number and cell area, respectively. The identity of the integrins engaged by the peptide-functionalized LB film was also examined. Many of the integrins, heterodimeric cell surface receptors ($\alpha_x\beta_y$), recognize the RGD motif; accordingly, the relationship between GRGDSP presentation and the identity of α- and β-subunits were explored using antibody-based inhibition assays.

2. Materials and methods

2.1. Fabrication of supported Langmuir–Blodgett films

The following sequences were synthesized as described elsewhere [32]:

$(C_{16})_2$–Glu–C_2–KAbuGRGDSPAbuK referred to as $(C_{16})_2$GRGDSP;
$(C_{18})_2$–Glu–C_2–KAbuGRGDSPAbuK referred to as $(C_{18})_2$GRGDSP;
$(C_{18})_2$–Glu–C_2–DPKAbuKGGRAbuS referred to as $(C_{18})_2$scr (GRGDSP);
$(C_{18})_2$–Glu–C_2–PHSRN referred to as $(C_{18})_2$PHSRN;
$(C_{16})_2$–Glu–C_2–KAbuGRGDSPAbuK–C_2–Glu–$(C_{16})_2$ referred to as $(C_{16})_2$GRGDSP$(C_{16})_2$;
$(C_{18})_2$−Glu–C_2–KAbuGRGDSPAbuK–C_2–Glu(C_{18}) referred to as $(C_{18})_2$GRGDSP$(C_{18})_2$;
$(C_{18})_2$–Glu–C_2–KDAbuSGAbuGRPK–C_2–Glu$(C_{18})_2$ referred to as $(C_{18})_2$scr(GRGDSP)$(C_{18})_2$, signifying a scrambled sequence.

Before assays were performed, mica disks, 15 mm in diameter, were cut, cleaved, and washed with chloroform, methanol, and MQ water. LB film depositions were done on a KSV 5000 LB system (KSV Instruments, Helsinki, Finland). Predetermined volumes (80–100 μl) of prepared solutions of amphiphilic molecules (~1.0 mg/ml) were spread on the air–water interface and allowed to evaporate for 15 min. All the depositions were done at 40 mN/m. Deposition speed for both the up and down strokes was 1 mm/min. 1,2-Distearoyl-sn-glycero-3-phosphatidylethanolamine (DSPE) (Avanti Polar Lipids, Inc., Alabaster, AL) layer was deposited first on the upstroke to make mica surfaces hydrophobic. The second layer with peptide amphiphiles and their mixtures with PEG (polyethylene glycol with molecular weight 120 covalently linked to DSPE) (Avanti Polar Lipids, Inc., Alabaster, AL), was deposited on the down stroke. Transfer ratios for both layers were calculated to be in the range 0.8–1, indicating that monolayers were deposited on mica surfaces with minimal disruption. The resulting supported membranes were transferred into glass holders under water. Care was taken to avoid exposing the surface to air, as they rearrange to form multilayers [35]. Before cell assays were performed, the MQ water in which the LB surfaces had been deposited was exchanged for a medium that would support cell adhesion (i.e., osmotically acceptable). All assays were performed in endothelial cell basal medium-2 (EBM-2), purchased from Clonetics, Inc., supplemented with 0.1% bovine serum albumin (BSA) (EBM-2 + 0.1% BSA). The glass holders with the LB films were placed in a CoStar® (VWR, Inc.) 12-well plates and incubated at 37 °C and 5% CO_2 for 2 h, prior to starting the assay.