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Effects of Supported Lipid Monolayer Fluidity on the Adhesion of Hematopoietic Progenitor Cell Lines to Fibronectin-Derived Peptide Ligands for $\alpha 5\beta 1$ and $\alpha 4\beta 1$ Integrins

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Abstract

Mimicking the *in vivo* stem cell niche to increase stem cell expansion will likely require the presentation of multiple ligands. Presenting ligands in fluid supported lipid monolayers (SLMs) or bilayers (SLBs) allows for ligand diffusion to complement the arrangement of cell receptors, as well as cell-mediated ligand rearrangement and clustering. Cells in tissues interact with ligands presented by other cells and the extracellular matrix (ECM), so it will likely be beneficial to present both cell-associated and ECM-derived ligands. A number of investigators have incorporated cell-membrane-associated ligands within fluid surfaces and several groups have shown that these ligands cluster beneath the cells. However, few studies have investigated cell adhesion to ECM-derived ligands in fluid surfaces. Fibronectin is an important ECM component in many tissues, including the hematopoietic stem cell niche. We examined adhesion of the M07e and THP-1 hematopoietic progenitor cell lines to fibronectin-derived peptide ligands for the $\alpha 5\beta 1$ (cyclic and linear RGD) and $\alpha 4\beta 1$ (cyclic LDV) integrins, as well as the heparin-binding domain (HBD), presented as lipopeptides in fluid and gel SLMs. M07e cells adhered more avidly than THP-1 cells to all of the lipopeptides in fluid and gel surfaces. Adhesion of both cell lines to all peptides was less avid in fluid vs. gel SLMs. Adhesion to cyclic LDV (cLDV) and cRGD was similar on gel SLMs for both cell lines. In contrast, adhesion to cLDV was less extensive than to cRGD in fluid SLMs, especially for M07e cells. Adhesion to linear RGD was less avid than to cRGD or cLDV, and decreased to a greater extent in fluid SLMs. Human aortic endothelial cells adhered to cRGD in fluid SLMs and remained viable for at least 24 h, but did not spread. We also showed additive THP-1 cell adhesion to cLDV and linear RGD lipopeptides presented in a fluid SLM. Although DOPC (dioleoyl phosphatidyl choline) SLMs are not sufficiently stable for long-term cell culture studies, our results and those of others suggest that fluid SLMs are likely to be useful for presenting multiple ligands and for mimicking short-term interactions in the stem cell niche.

Introduction

Interactions with accessory cells and the extracellular matrix (ECM) provide cues for the regulation of cell proliferation and differentiation *in vivo*. This has led to the hypothesis that mimicking the *in vivo* environment or niche would facilitate stem cell self-renewal and/or controlled maturation in culture¹. Mimicking the niche is complicated due to the large number of cell adhesion molecule (CAM) ligands and growth factors that comprise the niche. For

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example, ca. 15 different ECM components and 20 different growth factors, cytokines and chemokines - many of which are presented in both bound and soluble form - have been implicated in the hematopoietic stem cell niche ². While it is unlikely that all of the *in vivo* interactions must be mimicked, it is very likely that multiple ligands will have to be presented. The distribution of ligands on the surface is also likely to be important. Differentially patterning surfaces with one or more ligands is problematic, especially since the optimal patterns are not likely to be known *a priori* and because the patterns may need to be altered during culture ³.

Presentation of ligands within supported lipid monolayers (SLMs) or bilayers (SLBs) at temperatures above the lipid melt transition temperature (T_m) allows for ligand rearrangement to complement the pattern of cell receptors, and facilitates cell-mediated clustering and redistribution of receptors and ligands. Furthermore, supported phospholipid monolayers and bilayers exhibit low non-specific cell adhesion ^{4, 5} and can readily be used to simultaneously present multiple ligands. A number of studies have shown that ligands for cell-cell adhesion molecules and growth factor receptors cluster beneath cells when presented in fluid SLBs ⁶⁻⁹. Studies with immobilized CD58 (ligand for CD2) and immobilized EGF suggest that the dose-response curve for binding to surfaces with immobilized cell-cell adhesion ligands is shifted to lower ligand concentrations on SLBs with laterally diffusive vs. nondiffusive ligands ^{8, 10}. This is consistent with the idea that cell binding depends on the local ligand concentration.

Cells in tissues interact with other cells and ECM components. Thus, an ideal surface to modulate cell differentiation would include both cell-associated and ECM ligands. Unlike cell-membrane-associated ligands, ECM-associated ligands are normally rearranged over a much longer timescale ³. However, clustering of the integrin receptors for ECM-derived ligands can enhance cell adhesion and modulate signaling through the formation of focal adhesions ¹¹. Thus, it is likely that cell responses to ECM ligands will vary with SLB or SLM fluidity. However, few studies have evaluated the presentation of ECM ligands in fluid surfaces. Mouse L-cells and NIH 3T3 cells adhered to RGD-containing peptide amphiphiles in egg-PC-based fluid SLBs, but not to blank egg PC SLBs ¹². Although they did not spread initially, cells that adhered to RGD eventually spread after displacing the lipids to bind to the underlying glass substrate. Binding of non-spreading rat adult hippocampal progenitor (AHP) cells to the laminin-derived IKVAV peptide, which is not an integrin ligand, showed a similar dose-response curve when IKVAV was presented in a laterally mobile (linked to palmitoyl oleoyl phosphatidyl choline (POPC) in a fluid POPC bilayer) as compared to an immobile (linked to PLL-g-PEG) form ¹³. Differential cell adhesion to integrin ligands presented in fluid vs. gel SLB or SLM surfaces has not been evaluated.

Fibronectin is an important ECM component in many tissues including the hematopoietic stem cell niche in the bone marrow ², and contains multiple cell binding domains ^{14, 15}. In this study, we used the M07e and THP-1 human hematopoietic progenitor cell lines to evaluate the effects of ligand mobility on cell adhesion to fibronectin-derived ligands for the $\alpha 5 \beta 1$ integrin (high-affinity cyclic RGD and low-affinity linear RGD peptides ^{16, 17}) and the $\alpha 4 \beta 1$ integrin (cyclic LDV peptide ¹⁸). We also evaluated a peptide derived from the fibronectin C/H II heparin-binding domain ¹⁹, which has been shown to support the adhesion of hematopoietic stem and progenitor cells ¹⁵. Phospholipid SLMs containing phosphatidyl choline (PC) head groups were used to provide low non-specific binding. The lipid tail length and degree of saturation were varied to obtain gel (dipalmitoyl PC (DPPC), $C_{16:0}$, $T_m = 42^\circ\text{C}$) and fluid (dimyristoyl PC (DMPC), $C_{14:0}$, $T_m = 24^\circ\text{C}$; dioleoyl PC (DOPC), $C_{18:1}$, $T_m = -20^\circ\text{C}$) cell culture surfaces. Human aortic endothelial (HAE) cells were used to confirm the differences in ligand mobility, as evidenced by the observation that HAE cells were able to spread on RGD presented within DPPC SLMs, but not within DOPC SLMs. In contrast to previous reports for cell-membrane-associated ligands, we found that M07e and THP-1 cell adhesion to fibronectin-derived peptides decreased with increasing fluidity in a cell-type- and ligand-dependent manner.

Methods

Cell culture

M07e cells (DSMZ, Germany) were adapted from 20% FBS to 10% FBS and maintained in IMDM medium (Sigma, St. Louis, MO) supplemented with 10% FBS (Hyclone, Logan, UT), and 10 ng/mL GM-CSF (Bayer Healthcare Pharma, Wayne, NJ). THP-1 cells (ATCC, Manassas, VA) were maintained in RPMI medium (Sigma) supplemented with 2.5 g/L glucose and 10% FBS. Both cell types were cultured in a 5% CO₂ incubator at 37°C and were used during the exponential growth phase for all assays. HAE cells (Lonza, Basel, Switzerland) were cultured in endothelial growth medium with BulletKit-2 containing 2% FBS (Lonza, CC-3162). HAE cells were used between passage 6 and 8 and were allowed to reach ~70% confluence before trypsinizing for culture on surfaces.

Lipopeptide synthesis

Standard Fmoc solid-phase peptide synthesis strategies were employed to synthesize all peptides and lipopeptides, as described ⁴. The peptide sequences included GC*RGDWC*GY (cRGD, * indicates point of cyclization), YGGRGDSP (LinRGD), GC*WLDVC*GY (cLDV), and SEPLIGRKITY (HBD). Rink Amide AM or Rink Amide MBHA resin (NovaBiochem, San Diego, CA) was used for synthesis. For the lipopeptides, the spacer molecule Fmoc-undecapolyethylene glycol-acid (PEG₆₀₀, NovaBiochem, San Diego, CA) and the lipid anchor succinic acid-dipalmitoyl glycerol (su-DPG) were sequentially conjugated to the peptide prior to cleavage from the resin ⁴. Cleavage products were precipitated twice in cold ether and dried under vacuum. Products were purified with reverse phase HPLC on either a C₁₈ (peptides) or C₄ (lipopeptides) column (Waters, Milford, MA). Molecular weight was confirmed with matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectroscopy (Applied Biosystems Voyager Pro DE, Foster City, CA) as described ⁴.

Octadecyltrichlorosilane coating and supported lipid monolayer (SLM) formation

SLMs were formed as described ⁴, with minor modifications. *Hydrophobic surfaces.* Glass slides (Fisher, Pittsburg, PA) were cleaned by immersion into 12 N HCl:Methanol (1:1) for 30 min followed by immersion in A.C.S. grade (95-98%) H₂SO₄ for 30 min. Slides were thoroughly washed in deionized water and dried in a 60°C oven after each acid cleaning step. Clean glass slides were immersed, while suspended in an ultrasonic water bath, for 1 min into a coating solution (45:3:2 hexadecane:carbon tetrachloride:chloroform containing 5 µL/mL octadecyltrichlorosilane (OTS); Sigma) that had been previously sonicated for 10 min. Three, 3 min ultrasonic chloroform rinses were performed and slides were washed with 18 MOhm water and dried in a 60°C oven. Slides were stored at room temperature until use. *Culture well construction.* 16-well chamber slides (Lab-Tek, Christchurch, New Zealand) were removed from their glass base and clamped onto OTS-treated glass slides. Silicone rubber gasket does not bind tightly to OTS-treated glass; thus, the glass side was selectively etched by injecting 5 N NaOH through the gasket track and incubating for 3 h at room temperature. The gasket track was rinsed with 18 MOhm water until neutral. A self-curing silicone rubber gasket (Silastic, Dow Corning, Midland, MI) was injected into the gasket track and allowed to cure overnight. *Vesicle preparation.* Various ratios of carrier lipid (DPPC, DMPC, or DOPC; Avanti Polar Lipids, Alabaster, AL) and lipopeptides in chloroform were mixed in glass vials. Chloroform was evaporated with a stream of nitrogen gas. Lipids were placed under vacuum overnight to ensure removal of residual chloroform. Lipids were resuspended at 0.5 mg/mL total lipid concentration in 18 MOhm water, heated to 50-55°C, and vortexed until all lipids were in suspension and the solution became cloudy. Suspensions were tip sonicated for 2 min until clear and immediately passed through a 0.22 µm filter (Millipore, Billerica, MA). *SLM formation.* 100 µL of filtered lipid suspension was loaded into each well of 16-well cassettes. The cassettes were placed in a pre-heated box that contained a low level of water to prevent

dehydration of the lipid surfaces and incubated in a 60°C oven. After 15 min, any bubbles formed along the edges were carefully removed and cassettes (in boxes with a low level of water) were returned to a 60°C oven for 1.5 h. Surfaces were washed 5 times with 200 μ L of 18 MOhm water and 3 times with 200 μ L of normal growth media. Cassettes were incubated at 37°C and used within 30 min for cell culture unless otherwise noted.

Cell adhesion assay

For visualization in cell adhesion assays, M07e, THP-1, or HAE cells were stained with Calcein AM (Molecular Probes, Eugene, OR). Calcein AM was added to the cell suspension (1×10^6 cells/mL) at a final concentration of 5 μ g/mL and incubated for at least 15 min at 37°C, vortexing every 5 min. Cells were centrifuged at $200 \times g$ and resuspended in normal growth medium at a concentration of 600,000 - 800,000 cells/mL for use in cell adhesion assays.

Normal-force-centrifugation cell adhesion assays were performed as described⁴. Briefly, cells were loaded into cassettes with SLM surfaces and returned to a 37°C incubator. After 1.5 h, pictures were taken to determine the number of cells in the wells using a fluorescence microscope (Leica DM IRB, Heidelberg, Germany). After 2 h, cassettes were placed into heat-sealable bags filled with phosphate buffered saline (PBS), inverted, and spun in a centrifuge at $30 \times g$ for 5 min to remove non-adherent cells. Cassettes were placed right-side up while under PBS, imaged, and analyzed using NIH Image J software (<http://rsb.info.nih.gov/ij/>) to determine the number of adherent cells.

For evaluation of SLM stability, surfaces were made as described above except that the cassettes were prepared aseptically. Prior to the adhesion assay, surfaces were incubated for extended time periods either with complete M07e cell medium or with PBS or X-VIVO 20 serum-free medium (BioWhittaker, Walkersville, MD), as indicated. Surfaces incubated with PBS or X-VIVO 20 were rinsed 3 times with complete M07e cell medium immediately prior to performing the adhesion assay.

For evaluation of HAE cell spreading at 2 and 24 h, surfaces were made as described above. Cassettes for 24-h culture were prepared aseptically. HAE cells were loaded onto surfaces and allowed to interact for the specified time. Thirty min prior to imaging on a fluorescence microscope (Nikon TE 2000, Tokyo, Japan), 20 μ L of stain (10 μ L/mL Calcein AM in PBS) was added to each well.

Adhesion competition assay

Fibronectin (Sigma; 20 μ L/mL in PBS containing 1% bovine serum albumin (BSA)) was adsorbed onto unmodified 16-well chamber slides (Lab-Tek) overnight. Cells were incubated with 100 μ M soluble peptide (cRGD, cLDV, or both) for 30 min prior to incubation on fibronectin-coated surfaces. Subsequent steps were performed as described for the cell adhesion assay.

Confocal microscopy imaging

Surfaces were made as described above, except that reusable clamped cassettes with a silicone rubber gasket⁴ were used to ensure easy disassembly of the cassettes. Cells were loaded and spun to the surface at $30 \times g$ for 1 min. After 3 h, cells were fixed with 4% formaldehyde for 15 min and subsequently permeabilized with Triton X-100 for 15 min. Surfaces were carefully washed with 0.15 M glycine in PBS after each step. Surfaces were then washed 3 times with 1% BSA in PBS. Three μ L of anti- $\alpha 5\beta 1$ antibody (Millipore, MAB1999) was added to each well for 15 min. Wells were washed 3 times each with 1% BSA in PBS and 1% BSA plus 1% goat serum in PBS. An Alexa Fluor® 488-conjugated goat-anti-mouse antibody (Molecular Probes, A11029, Eugene, OR) was added at 1 μ L/well and incubated overnight. After washing

the wells with 0.15 M glycine in PBS, cassettes were disassembled and a coverslip was placed over the lipid surfaces while immersed in water. The slides were stored in a refrigerator at 4° C prior to imaging on an upright confocal microscope (Leica DM RXE, Germany).

Fluorescence recovery after photobleaching (FRAP)

Surfaces were made as described above, except that individual 16-mm wells were glued to OTS-coated coverslips using self-curing silicone rubber gasket. DPPC (with and without cRGD-DPG) and DOPC:cholesterol SLMs were photobleached using a Mg lamp and imaged with a fluorescence microscope (Leica DM IRB). The area of interest was highlighted by using a diffusion filter at its smallest setting, resulting in the bleaching of a hexagonal area. Images were taken before, during (5-10 min), and after bleaching. Diffusion of DPPE labeled on the head group with nitro-benzoxadiazole (NBD) was monitored in 15-min increments for at least 1 h after bleaching. Diffusion of NBD-DPPE in DOPC SLMs was too rapid to achieve bleaching using the above procedure, so DOPC SLMs were also bleached using an Ar laser and imaged on an inverted confocal microscope (Leica DM IRE 2). The FRAP module in the Leica software was used to bleach a designated spot and analyze the fluorescence in that spot before and after bleaching in 0.4- to 6-sec increments.

Flow cytometry

Cells (~100,000) were washed twice with 0.5 mL PBS containing 0.05% w/v sodium azide and 0.1% BSA (PAB) followed by centrifugation (3 min at $290 \times g$) and supernatant decanting. The cells were incubated with 10 μ L of mouse anti-CD49d-PE-Cy5 or mouse anti-CD49e-PE antibody (BD Biosciences, San Jose, CA) for 30 min in the dark at room temperature. After staining, cells were washed twice with PAB, resuspended in 0.3 mL PAB, and analyzed on a Becton Dickinson LSRII flow cytometer using FACSDiva software (BD Biosciences). Isotype controls were performed to control for non-specific binding.

Results

Differences in lipid monolayer fluidity were confirmed via fluorescence recovery after photobleaching (FRAP) and endothelial cell spreading

DPPC SLMs containing 0.5 mol% NBD-DPPE plus 1.0 mol% cRGD-DPG did not exhibit fluorescence recovery after photobleaching. In contrast, DOPC SLMs containing 0.5-1.0 mol % NBD-DPPE plus 1.0 mol% cRGD-DPG showed very rapid fluorescence recovery in the presence or absence of M07e cells (data not shown).

HAE cells were cultured on SLMs consisting of DOPC or DPPC with or without 1 mol% cRGD-DPG. We have previously shown, using 125I-labeled cRGD-DPG, that the lipopeptide density in DPPC and DOPC:cholesterol SLMs increased linearly with the mol% of lipopeptide in the vesicles up to at least 2.5 mol%, with a surface density of 8.5 ± 1.3 pmol cRGD/cm² per mol% cRGD-DPG in DPPC SLMs^{4, 20}. Images taken after 2 and 24 h of incubation show that cells on DOPC or DPPC SLMs with no incorporated lipopeptide remained round (Figure 1A-D) and exhibited a low level of adhesion after normal-force centrifugation (Figure 2A). Further, HAE cells did not adhere to OTS-coated glass surfaces in the absence of SLMs (Figure 2A). Cells cultured on DOPC SLMs with 1 mol% cRGD-DPG (DOPC+cRGD) adhered to the surfaces (Figure 2A), but did not spread (Figure 1E, F). HAE cells cultured for 2 h on DPPC SLMs with 1 mol% cRGD-DPG (DPPC+cRGD) exhibited a spread morphology (Figure 1G) and by 24 h formed interconnected networks (Figure 1H). After 3 h, HAE cells on DPPC +cRGD were only slightly more spread than those on DOPC+cRGD (Figure 2B-E). However, confocal microscopy images revealed different $\alpha 5\beta 1$ integrin distributions on the two surfaces. Cells cultured on DPPC+cRGD exhibited an organized pattern of $\alpha 5\beta 1$ integrins with a substantial radial component extending from the cell body to the edges of the radial projections

(Figure 2B,C). In contrast, the $\alpha 5 \beta 1$ integrins in cells on DOPC+cRGD were less organized and exhibited more of a swirling pattern (Figure 2D,E).

Stability of cell adhesion to cRGD-containing SLMs

M07e cell adhesion to DPPC and DOPC SLMs containing 1 mol% cRGD-DPG was evaluated after incubation of the surfaces with serum-containing medium (IMDM plus 10% FBS) for extended time periods. Adhesion to cRGD-DPG in DPPC SLMs was similar to that for fresh surfaces after incubation for 12 h, and then gradually decreased with time (ca. 20% decrease after 4 days; Figure 3A). In contrast, adhesion to cRGD-DPG in DOPC SLMs decreased much more rapidly - by ca. 75% after 24 h. The time-dependent decrease in adhesion of M07e cells (incubated with complete medium) to cRGD-DPG in either DOPC or DPPC SLMs was much less extensive if the surfaces were pre-incubated with X-VIVO 20 serum-free medium or, especially, PBS, rather than in complete medium with 10% FBS (Figure 3B). This suggests loss of the cRGD-DPG lipopeptide via exchange with lipids in FBS. M07e cells showed little if any adhesion to control DOPC or DPPC SLMs or to OTS-coated glass even after incubation with medium containing 10% FBS for up to 168 h (Figure 3A).

The rapid loss in cell adhesion to cRGD-DPG lipopeptides in DOPC SLMs with prior incubation time indicates that DOPC SLMs are not suitable for long-term cell culture studies. Additional experiments were carried out to evaluate the suitability of DOPC SLMs for short-term studies to assess the effects of fluidity on cell adhesion. Short-term DOPC SLM integrity is supported by the observation that adhesion was similar for M07e cells incubated for 2 h on DOPC SLMs containing 1 mol% cRGD-DPG in either PBS plus 0.2% BSA or IMDM plus 10% FBS (data not shown). Further, M07e cells showed similar adhesion to DOPC SLMs containing 1 mol% cRGD-DPG when incubated on the SLMs in IMDM plus 10% FBS for either 2 or 4 h (data not shown). Thus, DOPC SLMs are suitable for the short-term cell adhesion studies described below, most of which involved incubation of cells on SLMs for 2 h.

M07e and THP-1 cell characterization

M07e and THP-1 cells bind tightly to fibronectin via both $\alpha 4 \beta 1$ and $\alpha 5 \beta 1$ integrins, as evidenced by the observation that soluble versions of both integrin ligands (cLDV and cRGD, respectively) were required to block adhesion to fibronectin (Figure 4A). The two cell lines are similar in size, with diameters of $11 \pm 2 \mu\text{m}$ for M07e cells and $13 \pm 2 \mu\text{m}$ for THP-1 cells (Figure 4B), and expressed similar levels of $\alpha 4$ and $\alpha 5$ integrins (Figure 4C).

M07e cells bind more avidly than THP-1 cells to lipopeptide mimics of fibronectin binding domains presented in immobile DPPC SLMs

M07e and THP-1 cell adhesion to DPPC SLMs increased with increasing lipopeptide density in a cell- and ligand-dependent manner until a plateau was reached (Figure 5). Neither cell line spread on surfaces containing any of the lipopeptides (data not shown). Similar adhesion trends for the different ligands ($\text{cRGD} \approx \text{cLDV} > \text{LinRGD} > \text{HBD}$) were observed for M07e and THP-1 cells. However, M07e cells exhibited greater avidity for all of the ligands, as evidenced by the 2- to 5-fold lower ligand concentrations required to support 50% cell adhesion. Both cell lines bound most avidly to cRGD-DPG and cLDV-DPG, with plateaus at ca. 90% adherent cells. Similar adhesion to cRGD-DPG and cLDV-DPG for both cell lines is consistent with the observation that soluble cRGD and cLDV were both required to block binding to fibronectin for each cell line (Figure 4A). Similar to our results for the KG-1a human hematopoietic progenitor cell line⁴, binding to LinRGD-DPG was much less avid than that to cRGD-DPG for both cell lines (Figure 5). This is consistent with our observation that 100 μM soluble LinRGD only minimally decreased THP-1 cell binding to DPPC SLMs with 1 mol% cRGD and decreased binding to DPPC SLMs with 2.5 mol% LinRGD by ca. 60%, while 100 μM soluble cRGD completely blocked binding to both surfaces (data not shown). M07e cell binding

to HBD-DPG reached a similar plateau (ca. 75% adhesion; adhesion was similar for 5 mol% HBD-DPG (data not shown)) as binding to LinRGD-DPG, although a greater HBD ligand density was required. In contrast, THP-1 cells did not adhere to SLMs containing HBD-DPG.

M07e and THP-1 cell adhesion to lipopeptides is decreased in fluid SLMs

As was the case for DPPC SLMs, M07e cells adhered more avidly than THP-1 cells to all of the lipopeptides presented in fluid DMPC SLMs and DOPC SLMs (Figure 6A-C vs. Figure 6D-F). Adhesion to all of the lipopeptides was less avid on fluid as compared to gel SLMs for both cell lines, and the decrease was greater for THP-1 cells. In contrast to the DPPC surfaces, adhesion to cRGD-DPG (Figure 6A,D) was greater than that to cLDV-DPG (Figure 6B,E) on fluid SLMs, especially for M07e cells. Neither cell line adhered to HBD-DPG on fluid surfaces (data not shown). Thus, the avidity rank order for the other ligands (cLDV > LinRGD > HBD) was maintained for the DMPC and DOPC SLMs. Adhesion to the less effective LinRGD $\alpha 5\beta 1$ integrin ligand (Figure 6C,F) was decreased to a greater extent than that to the more effective cRGD ligand. This was especially true for M07e cells, which showed very robust adhesion to cRGD-DPG even on fluid SLMs (Figure 6A). The $\alpha 5\beta 1$ integrin distributions on THP-1 cells cultured for 3 h on fluid and gel SLMs incorporating cRGD-DPG (Figure 7) were distinct from those observed for HAE cells (Figure 2B-E), but there were still differences between the patterns on gel vs. fluid SLMs. THP-1 cells on DPPC+cRGD exhibited a punctate distribution of $\alpha 5\beta 1$ integrins across the cell (Figure 7A). In contrast, the $\alpha 5\beta 1$ integrins were primarily clustered at the periphery for cells on DOPC+cRGD (Figure 7B).

Cell adhesion to lipopeptides in DOPC SLMs was less than or equal to that in DMPC SLMs. Lower adhesion to ligands in DOPC SLMs could be due to the lower packing density of monounsaturated oleate chains vs. saturated myristate chains because the same mol% lipopeptide would correspond to a lower ligand density in pmol/cm². In order to correct for differences in the packing density of the different carrier lipids, we used the approach recommended by Nagle and Tristram-Nagle to estimate single lipid volumes in SLBs²¹. For the same mol% lipopeptide we estimate that DMPC SLMs and DOPC SLMs, respectively, would contain 80% and 66% as much lipopeptide as DPPC SLMs. The value of 12.6 pmol cRGD/cm² calculated for 1 mol% cRGD-DPG in DPPC SLBs is similar to the previously measured value of 8.5 ± 1.3 pmol/cm² for cRGD-DPG in a DPPC SLM⁴. Correcting for the lipid packing density essentially eliminated the differences in cell adhesion between fluid DMPC SLMs and DOPC SLMs, but cell adhesion was still much greater to lipopeptides presented in gel DPPC SLMs (Figure S1).

Incorporation of multiple ligands into supported lipid monolayer surfaces

The LinRGD-DPG and cLDV-DPG lipopeptides are ligands for $\alpha 5\beta 1$ and $\alpha 4\beta 1$ integrins, respectively. Incorporating both LinRGD-DPG (1.0 mol%) and cLDV-DPG (0.1 mol%) into the same DOPC SLM increased M07e cell adhesion by at least an additive amount compared to that for the individual lipopeptides (Figure 8).

Discussion

Many different culture surface chemistries have been used to evaluate cell adhesion to peptide ligands for $\alpha 5\beta 1$ and $\alpha 4\beta 1$ integrins^{4, 12, 22-28}, but very few of these surfaces have allowed for the lateral diffusion of the integrin ligands. We have evaluated the effects of integrin ligand lateral mobility on cell adhesion using phospholipid SLMs that differ in lipid tail composition. The use of PC head groups for all three lipids ensured that differences in ligand mobility were evaluated under conditions with the same surface chemistry. Ligand mobility in DOPC SLMs was confirmed by FRAP and also by the lack of spreading (Figure 1E,F) and the less organized structure of $\alpha 5\beta 1$ integrins (Figure 2D,E) for HAE cells cultured on DOPC SLMs with 1 mol

% cRGD-DPG, as well as extensive long-range clustering of $\alpha 5 \beta 1$ integrins around the periphery of THP-1 cells cultured on DOPC+cRGD (Figure 7B). These observations are consistent with reports showing that ligands for cell-cell adhesion molecules and growth factor receptors cluster beneath cells when presented in fluid SLBs⁶⁻⁹. Although HAE cells did not spread on DOPC SLMs containing cRGD-DPG, they adhered and remained viable for at least 24 h. In contrast, HAE cells spread extensively on DPPC SLMs containing cRGD-DPG, which is consistent with observations for human umbilical vein endothelial cells on the same surfaces⁴, and exhibited an organized distribution of $\alpha 5 \beta 1$ integrins beneath the cells (Figure 2B,C). One advantage of using SLMs to evaluate laterally mobile cell adhesion ligands is that cells do not adhere to the OTS-coated glass substrate. Thus, cells cannot spread on fluid RGD-presenting SLMs by displacing surface lipids to adhere to the substrate, as has been observed for egg PC SLBs on glass substrates¹².

High-avidity binding to cRGD and cLDV lipopeptides presented in DPPC SLMs for both cell types (Figure 5) is consistent with previous reports of avid binding to cRGD and cLDV presented in immobile systems^{4, 23-26, 29-32}. Much less avid binding to LinRGD-DPG is also consistent with prior reports^{4, 31, 33}. Cell adhesion to the fibronectin heparin-binding domain has been less extensively studied. Maximal adhesion of M07e cells to HBD-DPG in DPPC SLMs was similar to that for LinRGD-DPG, although the dose-response curve was not as steep (Figure 5A). In contrast, THP-1 cells showed essentially no adhesion to HBD-DPG in gel SLMs (Figure 5B).

Adhesion to all ligands was less avid on fluid surfaces for both M07e and THP-1 cells, but the extent of the decrease was cell- and ligand-specific. Similar to that for the DPPC SLMs, M07e cells adhered more avidly than THP-1 cells to all of the ligands presented in fluid SLMs (Figure 6). In contrast to similar adhesion to cRGD and cLDV in DPPC SLMs, both cell types adhered more avidly to cRGD than cLDV in fluid SLMs. Adhesion to LinRGD and HBD was decreased to very low levels in fluid surfaces for both cell types. After correcting for the lower lipid packing density in DOPC vs. DMPC SLMs (Figure S1), there does not seem to be any effect of the melt transition temperature in fluid surfaces. M07e cell adhesion to cRGD in fluid SLMs decreased to a lesser extent than for any of the other cell-ligand combinations. Interestingly, HAE cell adhesion to cRGD in fluid vs. DPPC SLMs (Figure 2A) decreased to a similar extent as that for M07e cells (Figure 6A). Clustering of integrin ligands under cells could decrease the effective surface concentration of ligands in the remaining surface, thereby decreasing the number of cells that could adhere to a fluid vs. gel surface with the same mol% lipopeptide. Decreased adhesion to integrin ligands in fluid surfaces contrasts with greater Jurkat T cell adhesion to CD58 (ligand for CD2) and greater MCF-10A cell adhesion to EGF presented as laterally diffusive ligands in SLBs^{8, 10}. These differences may be due to differences in the ligand binding properties. CD58 (LFA-3)/CD2 binding is characterized by large k_{off} and K_d values and GPI-LFA-3-FITC present in cell contact areas remained mobile, as evidenced by nearly complete recovery after photobleaching⁶. AHP cells showed similar binding avidity to the ECM-derived non-integrin ligand IKVAV when presented in a fluid SLB or immobilized via PEG to poly(L-lysine)¹³. This behavior is most similar to what we observed for M07e cell adhesion to cRGD. The cell- and ligand-dependent differences in adhesion to diffusible vs. immobile ligands that we and others have observed suggest that each cell-ligand combination will have to be examined experimentally. The greater adhesion to cRGD and cLDV that we observed for M07e vs. THP-1 cells cannot be attributed to differences in the number of cell surface integrins or the integrin surface density because the levels of $\alpha 4 \beta 1$ and $\alpha 5 \beta 1$ integrins and the cell size were similar for both cell types (Figure 4).

This study probes the interactions between integrin-ligand pairs by presenting ligands in surfaces that differ in their resistance to forces exerted by integrins and the cytoskeleton. Integrins are known to exert tension on the surrounding environment, and matrix compliance

has been shown to affect stress fiber formation, the stability of focal adhesion complexes, and cell functions such as spreading and motility³⁴. Fluid and gel SLMs are analogous to polymer films with very low Young's modulus (very soft surfaces) and high modulus, respectively. Differences in the lateral diffusivity of lipopeptides would be expected to alter cell-ligand forces in a manner analogous to differences in substrate compliance. Due to the low resistance, cells exhibit very little spreading on soft surfaces³⁵⁻³⁸. This is consistent with the lack of spreading by HAE cells on cRGD-DPG presented in DOPC SLMs (Figure 1E,F). Although cell traction forces cannot be exerted onto fluid SLMs, we believe that the analogy with very soft surfaces is relevant. Cells on very soft surfaces are not able to fully extend, and this is similar to the lack of cell extension on fluid SLMs. Also, there will be some resistance to the movement of lipopeptides within fluid SLMs, especially if the lipopeptides cluster together, as suggested by the image in Figure 7. Two recent studies indicate that cells adhere much less extensively to soft ECM-coated surfaces. Schneider et al.³⁵ found that only 25% as many chondrosarcoma cells adhered to soft (1-5 kPa) vs. more rigid (200 kPa) multilayer poly(L-lysine)/hyaluronic acid surfaces with an outer layer of hyaluronic acid. Guo et al.³⁹ showed similar differences in cell adhesion over a much smaller range of Young's modulus; 3-fold more Balb/c 3T3 cells adhered to collagen-coated substrates with a modulus of 8 as compared to a modulus of 3 kPa. Lower cell adhesion to softer surfaces is consistent with the observation that a lower force is required to peel cells off of soft surfaces (reviewed in³⁴) and may be attributed to fewer, smaller, and more transient focal adhesions on soft surfaces^{38, 39}. Lower adhesion forces and transient focal adhesions may also explain the less extensive adhesion to cRGD-DPG that we observed for HAE cells on fluid SLMs (Figure 2A), as well as the lower M07e and THP-1 cell adhesion to lipopeptide ligands for $\alpha 5\beta 1$ and $\alpha 4\beta 1$ integrins on fluid SLMs (Figure 6).

Although DOPC-based SLMs are not suitable for long-term studies due to loss of binding activity (Figure 3A), they are useful for short-term studies to evaluate effects of fluidity on cell adhesion and activation. Loss of binding activity with pre-incubation appears to be associated with lipopeptide exchange with serum lipids because SLM stability increased with decreasing serum content in the pre-incubation medium (Figure 3B). Furthermore, cell adhesion to lipopeptides in DOPC SLMs appears to protect the lipopeptide ligands from exchange with serum lipids. For example, M07e cell adhesion did not decrease between 2 and 4 h on a DOPC SLM containing 1% cRGD-DPG (data not shown). Also, HAE cells loaded onto DOPC SLMs containing 1% cRGD-DPG at time zero decreased by only 25% after 24 h in medium containing 2% FBS (Figures 1F and 2A). The extended stability may be due to the lower FBS content of the HAE cell medium (Figure 3B). Finally, M07e and THP-1 cell adhesion to cRGD and cLDV in DMPC SLMs was similar to that in DOPC SLMs (Figures 6 and S1), both of which are fluid surfaces. In contrast, the stability of DMPC SLMs appears to be much closer to that of DPPC SLMs than DOPC SLMs (data not shown).

Our results and those of others suggest that fluid SLMs would be effective for mimicking short-term cell-ECM and cell-cell interactions present in a stem cell or tissue niche. We have demonstrated that both nonspreading and spreading cells adhere to ligands for $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins presented in fluid SLMs. Dose-dependent adhesion to cRGD has also been reported for egg PC SLBs¹². We showed that ligands which interact with different integrins can be incorporated into the same fluid SLM and cooperate to increase cell adhesion (Figure 8). This is similar to additive adhesion reported for cLDV and cRGD presented in PEG-based surfaces²⁵. Others have shown that cells adhere to EGF and the cell-cell adhesion molecules E-cadherin and CD58 when presented in fluid SLBs⁷⁻⁹. Although cell adhesion to integrin ligands requires greater ligand densities in fluid SLMs, the simultaneous presentation of cell-cell adhesion ligands is likely to result in cooperative adhesion. It is possible that integrin ligand densities that do not support cell adhesion in the inverted centrifugation assay will be sufficient to provide a synergistic increase in cytokine-mediated signal transduction⁴⁰⁻⁴³. Furthermore,

large amounts of ligands can be incorporated in lipid-based systems. For example, Thid *et al.* were able to obtain twice the IKVAV ligand density in POPC SLBs as compared to that for PLL-*g*-PEG¹³. Anchorage-dependent HAE cells adhered to cRGD and remained viable on DOPC surfaces for 24 h, but did not spread (Figures 1 and 2A). If necessary, patterned posts or ECM components can be included within a fluid surface to allow for cell spreading, while retaining other ligands in a fluid surface^{9, 44}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Images taken before normal-force centrifugation of HAE cells cultured on DPPC or DOPC SLMs with or without 1 mol% cRGD-DPG. Images were taken with a 10X objective after 2 or 24 h of incubation on DOPC (A&B), DPPC (C&D), DOPC + 1 mol% cRGD-DPG (E&F), or DPPC + 1 mol% cRGD-DPG (G&H).

Figure 2.

HAE cell adhesion to DPPC and DOPC SLMs with or without 1 mol% cRGD-DPG and $\alpha 5 \beta 1$ integrin distribution on SLMs containing 1 mol% cRGD-DPG. HAE cell adhesion after culture for 2 h (hatched bars) and 24 h (grey bars) followed by normal-force centrifugation (A). $\alpha 5 \beta 1$ staining of HAE cells cultured on DPPC (B&C) and DOPC (D&E) surfaces with 1 mol% cRGD-DPG. Cells in (B-E) were allowed to adhere for 3 h before they were fixed and stained. Images were taken with an oil-immersed 100X objective.

Figure 3.

Stability of M07e cell adhesion to cRGD-DPG lipopeptides presented in DPPC and DOPC SLMs. Surfaces consisting of OTS (circles), DPPC (squares), DPPC + 1.0 mol% cRGD-DPG (diamonds), DOPC (triangles), and DOPC + 1.0 mol% cRGD-DPG (inverted triangles) were incubated for the specified time period in complete M07e cell medium, after which M07e cells were added to the surfaces and incubated for 2h to evaluate cell adhesion (A). DPPC (closed symbols) and DOPC (open symbols) SLMs containing 1.0 mol% cRGD-DPG were incubated with PBS buffer (circles), X-VIVO 20 serum-free medium (squares), or IMDM plus 10% FBS (triangles) for the specified time period before rinsing the wells with complete M07e cell medium and evaluating M07e cell adhesion (B).

Figure 4.

M07e and THP-1 cell interactions with fibronectin. Soluble competition of M07e cell (hatched bars) or THP-1 cell (grey bars) adhesion to FN with 100 μ M soluble peptides cRGD, cLDV, or cRGD + cLDV (A). Coulter Multisizer histograms of the M07e (solid line) and THP-1 (dashed line) cell diameter distributions (B). Relative expression levels of α 4 (CD49d) and α 5 (CD49e) integrins on the surface of M07e (hatched grey) and THP-1 (grey bars) cells measured using flow cytometry (C).

Figure 5.

Dose-dependent cell adhesion to lipopeptides presented in DPPC SLMs. M07e cells (A) and THP-1 cells (B) showed similar adhesion trends on DPPC SLMs incorporated with increasing amounts of cRGD-DPG (circles), LinRGD-DPG (inverted triangles), cLDV-DPG (triangles), and HBD-DPG (diamonds). However, M07e cells adhered more avidly than THP-1 cells to all of the lipopeptides.

Figure 6. M07e (A-C) and THP-1 (D-F) cell adhesion to cRGD-DPG (A&D), cLDV-DPG (B&E), and LinRGD-DPG (C&F) in gel and fluid surfaces. Lipopeptides were incorporated at the indicated molar percentages into SLMs formed with DPPC (circles), DMPC (triangles), or DOPC (inverted triangles) carrier lipids.

Figure 7. $\alpha 5 \beta 1$ integrin staining of THP-1 cells on DPPC (A) and DOPC (B) SLBs with 1 mol% cRGD-DPG. Images represent sections of one cell on each surface from point of contact (i) to cell midsection (iii). Cells were allowed to adhere for 3 h before they were fixed and stained. Images were taken with an oil-immersed 100X objective.

Figure 8. M07e cell adhesion to cLDV-DPG (0.1 mol%) and/or LinRGD-DPG (1.0 mol%) incorporated into DOPC SLMs.