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The microenvironment of immobilized Arg-Gly-Asp peptides is an important determinant of cell adhesion

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Abstract

This paper uses self-assembled monolayers on gold as a model system to demonstrate that the attachment and spreading of Swiss 3T3 fibroblasts depends strongly on the microenvironment of immobilized RGD peptides. This work utilized monolayers that present mixtures of Arg-Gly-Asp peptides, which are ligands for cellular integrin receptors, and oligo(ethylene glycol) groups, which resist the nonspecific adsorption of protein. The microenvironment of the peptide ligands was controlled by altering the length of the surrounding oligo(ethylene glycol) groups on the monolayer. By using thiols that present either tri-, tetra-, penta-, or hexa(ethylene glycol) units, the average distance separating the glycol groups and the peptide ligand is altered while the structure and properties of the background remain unchanged. Cell attachment to monolayers presenting a fixed density of peptide decreased as the length of the oligo(ethylene glycol) group increased. The average projected area of attached cells showed a similar trend. At lower densities of immobilized peptide, decreases in both cell attachment and projected cell area were more pronounced. Attachment and spreading did not depend on density of peptide on monolayers presenting tri(ethylene glycol) groups, but showed a high sensitivity to density of ligand on monolayers presenting longer glycol oligomers. Experiments that used a soluble peptide to inhibit the attachment of cells to monolayers demonstrated that the strength of the cell–substrate interaction decreased on monolayers presenting longer glycol groups. Together, these results suggest that the microenvironment of the peptide ligand influences the affinity of the integrin–peptide interaction and that weaker interactions display a density-dependent enhancement of binding during cell attachment and spreading. This finding is an important consideration in studies that correlate biological function with the composition of ligands on a substrate. This finding also represents an important principle for the design of biologically active materials because it illustrates the degree to which the *presentation* of adhesion motifs can modify the response of mammalian cells.

Keywords: Self-assembled monolayer; RGD; Cell adhesion; Cell spreading; Model substrate

1. Introduction

The interactions between integrin receptors on the surface of mammalian cells and ligands within the extracellular matrix (ECM) regulate a diverse range of cellular functions, including adhesion, growth, differentiation, and motility [1–6]. In many cases, these ligands comprise short peptide sequences. The Arg-Gly-Asp (RGD) sequence, found within many ECM proteins, has been the most extensively studied of these motifs, and substrates that present this peptide have found widespread use in adhesion research [7–10]. Previous work using model substrates has shown that the morphology of attached cells is dependent on the density of immobilized RGD [11,12], but no studies have unambiguously demonstrated how the groups surrounding peptide ligands — which define the microenvironment of the peptides — influence cell adhesion and morphology. In this paper, we use self-assembled monolayers (SAMs) of alkanethiolates on gold that present Gly-Arg-Gly-Asp-Ser (GRGDS) peptide ligands as a model system to show that the number and morphology of attached Swiss 3T3 fibroblasts depend strongly on the microenvironment in which peptide ligands are presented. This finding is an important consideration in studies that correlate biological function with the composition of ligands on a substrate. This finding also represents an important principle

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for the design of biologically active materials because it illustrates the degree to which the *presentation* of adhesion motifs can modify the response of mammalian cells.

Substrates used for studies of integrin-dependent cell adhesion are usually prepared by allowing a layer of purified protein to adsorb from solution onto glass or tissue culture substrates. These substrates are important because they are easily prepared and resemble the extracellular matrix to which cells attach in vivo, but the structure of the protein film is heterogeneous. This heterogeneity arises from the number of orientations in which proteins can adsorb and the degree to which proteins can denature at the surface [13–16]. A consequence of this heterogeneity is that it is difficult to control (or even determine) what fraction of the ligands present in the adsorbed film can be recognized by receptors of attached cells. Strategies that use radioisotopic labeling can determine the density of protein on a substrate, but this and related techniques provide no information about the number of ligands that are accessible to cellular receptors or how the groups surrounding these ligands influence binding interactions.

Early model substrates substituted ECM protein with discrete peptide ligands in order to control better the interactions between the cell and the substrate. Brandley and Schnaar, for example, prepared polyacrylamide gels derivatized with an RGD peptide and showed that 3T3 fibroblasts could attach and spread on these substrates [17]. It was not possible, however, to determine the number of ligands that were accessible to the cells because a fraction of the peptides were located in the interior of the gel and were inaccessible to integrins. Moreover, those ligands that were accessible were presented in heterogeneous environments. Other model systems for adhesion had the same limitations, including copolymers grafted with RGD peptides [18–20], hydrogels derivatized with RGD [21–23], Langmuir–Blodgett films presenting peptide amphiphiles [24,25], and proteins covalently modified with RGD [26–29].

Massia and Hubbell first reported a class of substrates where the ligands were immobilized in a homogeneous environment [11,30]. These substrates were formed by the immobilization of Gly-Arg-Gly-Asp-Tyr peptides to alkylsiloxane monolayers on glass. By radiolabeling the peptide, they determined the density of peptide ligand that supported the adhesion of cells and the density at which the attached cells assembled focal adhesions and stress fibers. This approach provided excellent control over the structure of the substrate, but these substrates were not completely resistant toward the adsorption of protein and the remodelling of matrix by attached cells, making studies that require long-term cell culture difficult. Later work showed that model substrates presenting fluorinated polymers [19,20], alginate hydrogels [21], or poly(ethylene glycol) conjugates [22–25] were more effective at resisting the nonspecific adsorption of protein, but none of these systems presented ligands in a homogeneous manner.

Self-assembled monolayers (SAMs) of alkanethiolates on gold are a class of model substrates that are both structurally well-defined and completely inert toward the nonspecific adsorption of protein [31,32]. These monolayers form spontaneously upon the adsorption of alkanethiols from solution onto a clean surface of gold. Since the properties of a SAM depend upon the terminal functional group of the precursor alkanethiol, virtually any surface can be prepared using organic synthesis. Monolayers that present oligo(ethylene glycol) groups are highly effective at resisting the nonspecific adsorption of protein, making them especially well suited for studies of protein–ligand interactions [33]. In previous work, we synthesized alkanethiols terminated with the GRGDS peptide and prepared SAMs that presented mixtures of this peptide and tri(ethylene glycol) groups [34]. We showed that Swiss 3T3 fibroblast cells attached selectively to these substrates. Using a similar substrate, we showed that the morphology of bovine capillary endothelial cells depends strongly on the density of peptide in the monolayer and that these substrates resist the remodelling of ECM by attached cells, even after twenty-four hours in culture [12].

In this study, we use self-assembled monolayers to demonstrate the extent to which the microenvironment of immobilized peptide ligands influences the adhesion of Swiss 3T3 fibroblasts. We controlled the microenvironment of the ligand by changing the length of the surrounding oligo(ethylene glycol) groups (Fig. 1). By using thiols that present either tri-, tetra-, penta-, or hexa(ethylene glycol) units, the average distance separating the glycol groups and the peptide ligand is altered while the structure and properties of the background remain unchanged [35]. Our results demonstrate that, for a fixed density of peptide, increasing the length of the oligo(ethylene glycol) alkanethiol significantly decreases the efficiency of cell attachment and spreading. This work also shows that the microenvironment of peptide ligands influences the affinity of the polyvalent integrin–peptide interaction and that greater enhancements in cell attachment and spreading occur when the binding interaction is weak.

2. Materials and methods

2.1. Cells and reagents

Swiss Albino 3T3 cells (ATCC, Rockville, MD) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% bovine calf serum and penicillin/streptomycin. All cultures were maintained at 37°C in a humidified 10% CO_2 atmosphere. All media and reagents used

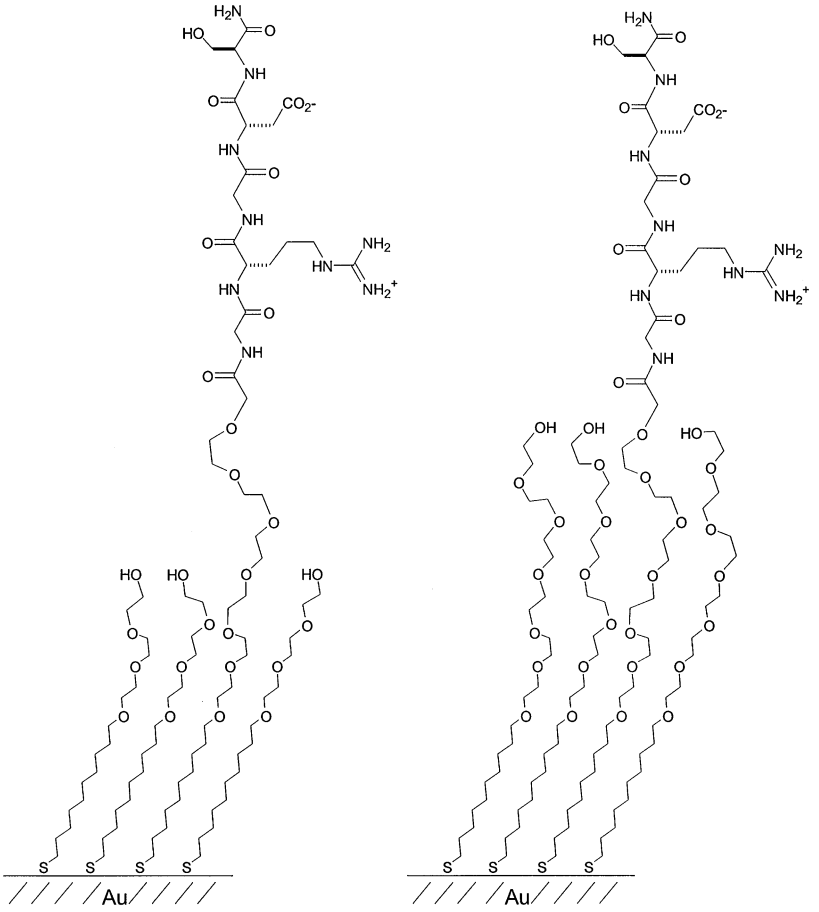

Fig. 1. Monolayers that present Gly-Arg-Gly-Asp-Ser peptides and oligo(ethylene glycol) groups were used as substrates for the attachment and spreading of Swiss 3T3 fibroblasts. The microenvironment of the peptide depends on the length of the ethylene glycol oligomer in the background. The shorter tri(ethylene glycol) group leaves the peptide ligand more accessible (left) than does the longer hexa(ethylene glycol) group (right).

in cell culture were obtained from Gibco BRL (Gaithersburg, MD). Monoclonal mouse anti-vinculin IgG, EDTA, cycloheximide, and ^{14}C-labeled formaldehyde were purchased from Sigma Chemical Company (Milwaukee, WI). Alexa-564 phalloidin and Alexa-488 goat anti-mouse IgG were obtained from Molecular Probes, Inc. (Eugene, OR). Dialysis tubing was purchased from Spectrum (Laguna Hills, CA).

2.2. Preparation of self-assembled monolayers

Substrates were prepared as described previously [36]. Titanium (1 nm) and then gold (12 nm) were evaporated onto glass coverslips. The coverslips were cut into pieces approximately 1 cm^2 in size and immersed in 0.4 ml of an ethanolic solution containing GRGDS alkanethiol conjugate **1** [37] and oligo(ethylene glycol) conjugate **2a, 2b, 2c,** or **2d** [38] (Fig. 2). All solutions were 1, 5, 10 or 25 μM in peptide and 1 mM in total thiol. After 5 h, the substrates were removed from the solutions, rinsed with absolute ethanol and dried under a stream of nitrogen. For convenience, we will refer to the monolayers used in this work by the mole fraction of peptide in the solution of alkanethiol from which the monolayers were prepared.

2.3. Radiolabeling of peptide–alkanethiol conjugate

Gly-Arg-Gly-Asp-Lys alkanethiol conjugate **3** was prepared as described previously [37] and labeled by reductive methylation with [^{14}C]-formaldehyde [39]. A solution of **3** (1 mM), [^{14}C]-formaldehyde (2 mM, 58 μCi/mmol), DTT (20 mM) and $NaBH_3CN$ (150 mM) in buffer (5 mM HEPES, pH 7.5, 100 μl) was shaken for 24 h at room temperature. The reaction mixture was diluted to a volume of 1 ml with water containing methanol (10%) and trifluoroacetic acid (0.1%) and dialyzed (MW cutoff 500) against the same solution. Lyophilization of the solution afforded the labeled peptide as a white solid. A specific activity of 27 μCi/mmol was determined using liquid scintillation counting.

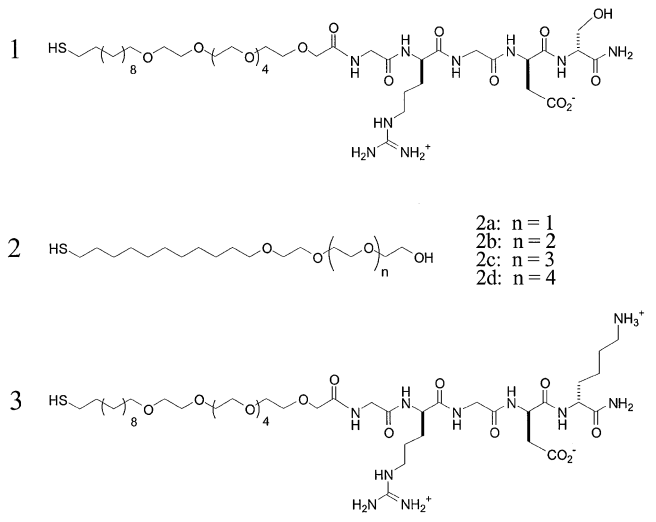

Fig. 2. Structures of alkanethiols used for the preparation of self-assembled monolayer substrates.

2.4. Determination of peptide surface concentration

SAMs prepared from solutions containing the ^{14}C-labeled peptide alkanethiol and oligo(ethylene glycol) alkanethiols (1, 5, 10 or 25 μM peptide; 1 mM total thiol) were exposed to a Molecular Dynamics phosphor imaging screen for one week. The screen was scanned, digitized, and analyzed with Image Quant software. A fixed area in each the digital image of each sample was traced, and the background activity for a region having the same area was subtracted to determine activity per unit area. Separate aliquots of the labeled peptide in ethanol (1, 0.5, 0.25, 0.125 and 0.0625 mM) were evaporated onto clean gold surfaces and counted as an internal standard.

2.5. Assay for cell attachment

Near confluent monolayers of cells were washed twice with (Ca^{2+}/Mg^{2+})-free Hank's balanced-salt solution (HBSS) and detached using 2 mM EDTA in (Ca^{2+}/Mg^{2+})-free HBSS. Serum-free culture medium was added, and the cells were centrifugated and resuspended in fresh culture medium. The cells were washed a second time with serum-free medium, and a fixed number of cells (40,000 cells in 2 ml culture medium) was plated onto substrates presenting the GRGDS peptide (1, 0.5 or 0.1%) among tri-, tetra-, penta-, or hexa(ethylene glycol) groups. After 5 h, the substrates were washed gently with Dulbecco's phosphate buffered saline (DPBS) and fixed for 30 min in 4% paraformaldehyde in DPBS. The number of cells attached per field was determined by counting cells on a 2×2 mm grid under $10\times$ magnification (Zeiss Axiovert 135). At least six adjacent fields were counted on each substrate, and the experiment was repeated on three separate occasions.

2.6. Measurement of cell spreading

The average spread area of fibroblasts attached to each monolayer was determined from digitized images of the cells as described previously [28]. Briefly, images of cells attached to monolayers were captured at 10X magnification using a Sony black & white CCD camera. The projected area of each cell was determined by tracing the outline of the cell and integrating this area in NIH Image. An object of known size was used to calibrate the measurements. Data points represent an average area of at least 50 cells on at least two different substrates.

2.7. Inhibition of cell attachment using soluble peptide

Cultures were washed twice with (Ca^{2+}/Mg^{2+})-free HBSS and detached using 2 mM EDTA in (Ca^{2+}/Mg^{2+})-free HBSS. The cells were resuspended in serum-free DMEM containing 1 mg/ml BSA and different concentrations of H_2N-Gly-Arg-Gly-Asp-Ser-$CONH_2$ (25 μM–1 mM). The cells were incubated at 37°C for 15 min and then placed onto monolayers presenting 1% GRGDS among tri-, tetra-, penta-, or hexa(ethylene glycol) groups. After an additional 2 h at 37°C, the substrates were washed gently with serum-free medium, and attached cells were counted as described above. Four fields were counted on each substrate, and at least two substrates were analyzed for each measurement. Attachment is reported as a percentage of cells attached to substrates in the absence of soluble peptide.

2.8. Immunofluorescence microscopy

Fixed cells were immersed in a 50 mM solution of NH_4Cl in Dulbecco's phosphate buffered saline (DPBS) for fifteen minutes and permeabilized with DPBS containing 0.3% Triton X-100 for 5 min. Cells were rinsed twice with DPBS containing 0.1% Triton X-100 (DPBST) and blocked for one hour in DPBS containing 10% goat serum. Focal adhesions were visualized by staining for one hour with monoclonal anti-vinculin IgG (1:300 in DPBS), followed by Alexa 488-conjugated goat anti-mouse IgG (1:200 in DPBS). Actin filaments were visualized by a 30 min exposure to Alexa 564-conjugated phalloidin (0.1 U/ml in DPBS). Substrates were mounted by inverting onto 5 μl of PBS containing 90% glycerol and 4% *n*-propyl gallate. The preparations were viewed using a 63X Plan-Apo objective (Zeiss).

3. Results

3.1. Microenvironment of peptide ligand does not alter density of ligand

The ratio of two alkanethiols present in a mixed monolayer is almost always different from the ratio of

alkanethiols in the solution from which the monolayer is prepared [40]. It was therefore necessary to determine the density of GRGDS peptide in each monolayer and to ensure that the density of peptide did not change with the length of the oligo(ethylene glycol) alkanethiol. We prepared monolayers from ethanolic solutions containing a mixture of ^{14}C-labeled Gly-Arg-Gly-Asp-Lys peptide alkanethiol conjugate and a tri-, tetra-, penta-, or hexa(ethylene glycol) alkanethiol conjugate. The density of labeled peptide on each of these substrates was determined by exposure to a phosphor screen. Fig. 3 illustrates the relationship between the length of the oligo(ethylene glycol) alkanethiol used in each solution and the amount of labeled peptide present in the corresponding monolayer. These data show that the amount of peptide-terminated alkanethiol present in a monolayer does not vary significantly with the length of the oligo(ethylene glycol) alkanethiol used to prepare the monolayer. The incorporation of peptide into monolayers prepared from solutions containing 0.1% peptide was also similar for each oligo(ethylene glycol) group (data not shown).

To quantitate the density of peptide on each monolayer, the intensity of signal from each substrate was compared to that generated by a series of concentrations of labeled peptide evaporated onto gold slides. Monolayers prepared from solutions containing 2.5% peptide (25 μM peptide alkanethiol, 1 mM total thiol) had an average surface density of peptide of 17 pmol/cm^2. Monolayers prepared from solutions containing 1, 0.5 and 0.1% peptide alkanethiol (v/v) had average densities of peptide of 7.5, 2.5 and 0.7 pmol/cm^2, respectively.

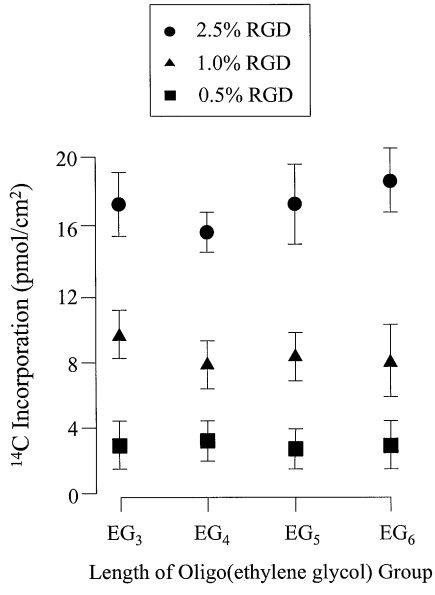

Fig. 3. The incorporation of ^{14}C-labeled Gly-Arg-Gly-Asp-Ser peptides into monolayers does not vary with the length of the oligo(ethylene glycol) group. Monolayers were prepared from solutions containing a ^{14}C-labeled Gly-Arg-Gly-Asp-Lys peptide alkanethiol conjugate (0.5, 1 or 2.5% by volume) and a tri-, tetra-, penta-, or hexa(ethylene glycol)-terminated alkanethiol (1 mM total thiol). The activity of each substrate was quantitated by imaging with a phosphor screen and compared to the activity of known amounts of peptide. Data points represent the average of three substrates, and error bars indicate one standard deviation from the mean.

3.2. Microenvironment of ligand influences cell adhesion

To evaluate the efficiency of cell adhesion to immobilized ligands in different microenvironments, we allowed cells to attach to substrates presenting the GRGDS peptide mixed with alkanethiols presenting either tri-, tetra-, penta-, or hexa(ethylene glycol) groups. Optical micrographs of representative substrates are shown in Fig. 4, and the average number of cells that attached to each substrate is shown in Fig. 5A. Fig. 5A shows that, for each of the three densities of peptide, the number of cells that attached to the substrate decreased as the length of the oligo(ethylene) glycol groups surrounding the peptides increased. For example, 160 ± 30 cells/field attached to a SAM presenting 1% GRGDS and tri(ethylene glycol) groups, while 100 ± 15 cells/field attached to a monolayer presenting 1% peptide among hexa(ethylene glycol) groups. The length of the oligo(ethylene glycol) group influenced attachment most dramatically at a peptide density of 0.1%. The average number of cells that attached to monolayers presenting this density of ligand and hexa(ethylene glycol) groups was 30-fold lower than the number of cells attached to monolayers presenting the ligand among tri(ethylene glycol) groups.

This figure also illustrates that cell attachment showed a stronger dependence on density of immobilized peptide as the length of the oligo(ethylene glycol) group increased. For substrates presenting tri(ethylene glycol) groups, the number of cells that attached did not vary significantly as the density of peptide decreased from 1 to 0.1%. Substrates presenting hexa(ethylene glycol) groups, by contrast, showed a 25-fold decrease in the number of cells that attached as the density of peptide decreased from 1 to 0.1%.

3.3. Microenvironment of ligand influences cell spreading

To determine the degree to which the length of the oligo(ethylene glycol) group influenced cell spreading, we measured the average projected area of cells attached to each substrate shown in Fig. 4. Since the number of cells that attached to each monolayer varied with the composition of the substrate, different numbers of substrates were examined for each data point. Fig. 5B shows that the projected cell area depends strongly on the microenvironment of the immobilized peptide when the density of peptide was 0.5 or 0.1%, but is less important when the density of peptide was 1%. The area of cells adherent to monolayers presenting GRGDS peptide at a density of 0.5%, for example, had an average projected area of 3300 μm^2 when mixed tri(ethylene glycol) groups but an

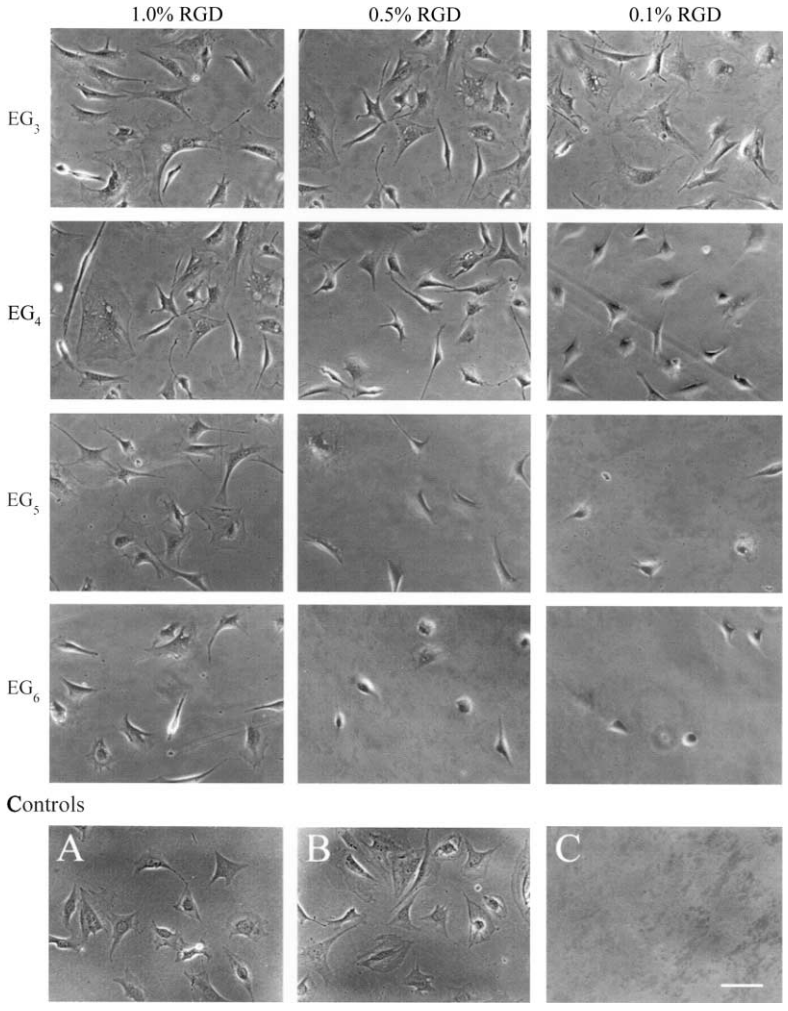

Fig. 4. Optical micrographs of cells attached to monolayers. Swiss 3T3 fibroblasts suspended in serum-free medium were added to substrates presenting the GRGDS peptide (0.1, 0.5 or 1%) mixed with alkanethiols presenting either tri-, tetra-, penta-, or hexa(ethylene glycol) groups. All photographs were taken at 20 × magnification after 5 h in culture. The control experiments show cell attachment to (A) a tissue culture dish, (B) fibronectin-coated glass and (C) a monolayer presenting tri(ethylene glycol) alone. The scale bar represents 120 μm.

average area of 1040 μm^2 when mixed with hexa(ethylene glycol) groups. The projected area of cells attached to SAMs presenting 1% peptide decreased only slightly over the same change in oligo(ethylene glycol) group.

The degree to which the density of immobilized peptide influenced cell spreading depended on the length of the oligo(ethylene glycol) group. The average projected cell area on substrates presenting tri(ethylene glycol) groups decreased from 3640 to 3040 μm^2 as the density of peptide decreased from 1.0 to 0.1%. The projected cell area on substrates presenting hexa(ethylene glycol) groups, however, decreased 5-fold over the same change in density of immobilized peptide. Cell attachment and spreading were not influenced by protein synthesis, since cultures treated with cycloheximide (20 μg/ml) before and during the experiment gave similar results (data not shown). These differences were also not due to changes in rate of cell attachment to different substrates, since longer incubation times did not alter cell attachment or spreading.

3.4. Microenvironment of ligand influences inhibition of cell attachment

We reasoned that the length of the oligo(ethylene glycol) group influenced cell attachment by changing the affinity of immobilized ligands for cellular integrins. To gain support for this interpretation and to examine the specificity of the cell–substrate interaction, we determined the concentration of soluble GRGDS peptide required to reduce the number of cells that attached by 50% (IC_{50}). We used monolayers presenting 1% peptide for these experiments because cells attached efficiently to substrates presenting this density of peptide among all four oligo(ethylene glycol) groups. Fig. 6 shows that the addition of soluble GRGDS (1 mM) to a suspension of cells completely inhibited attachment to all substrates. This figure also shows that value of the IC_{50} decreased as the length of the oligo(ethylene glycol) group increased. The IC_{50} for substrates presenting GRGDS among

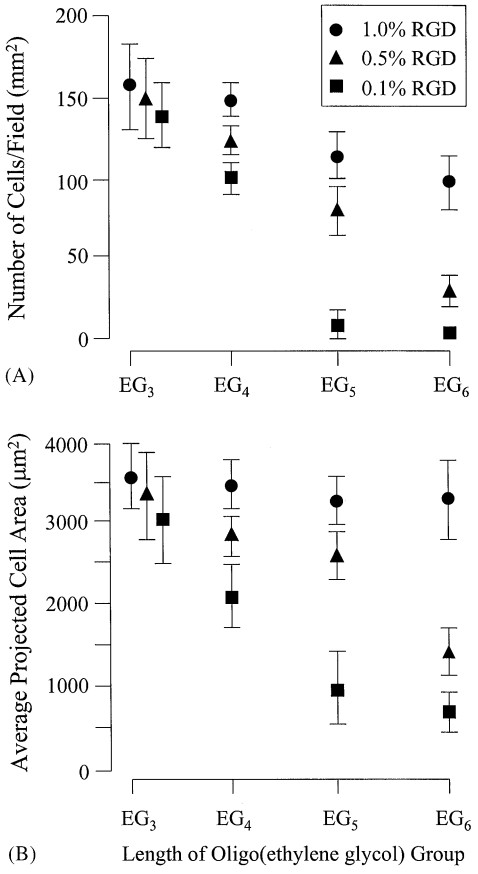

Fig. 5. (A) The number of cells that attach to self-assembled monolayers depends on the density of immobilized peptide and the length of the oligo(ethylene glycol) group surrounding the peptide. Cells were added to each of the monolayers described in Fig. 3, fixed after 5 h in culture, and counted at 10× magnification. The data are the average of three separate experiments. (B) The projected cell area of fibroblasts attached to monolayers described in (A). Data points represent the average area of at least 50 cells on at least two different substrates. Error bars indicate one standard deviation from the mean. Overlapping data points are offset horizontally for clarity.

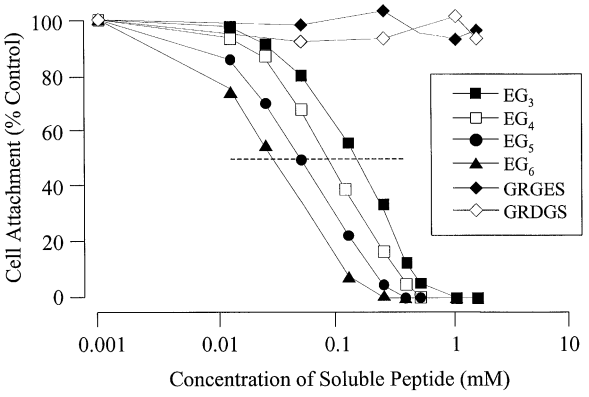

Fig. 6. Cell adhesion to monolayers is inhibited by soluble GRGDS-NH_2 peptide. Cells were incubated in serum-free media with concentrations of peptide ranging from 50 μM to 1 mM for 15 min at 37°C and added to monolayers presenting 1% GRGDS peptide among either tri-, tetra-, penta-, or hexa(ethylene glycol) groups (denoted as EG_3, EG_4, EG_5 and EG_6, respectively). After 2 h, attached cells were counted. Cell attachment to monolayers was not inhibited by the scrambled peptide GRDGS-NH_2 or by GRGES-NH_2 (denoted as GRDGS and GRGES in the figure legend). The dotted line represents 50% attachment and is provided as a guide. Data represent the average number of cells attached to at least two substrates The experimental error in each data point was less than 10%. Error bars are omitted for clarity.

tri(ethylene glycol) groups was 160 μM, while values for substrates presenting tetra-, penta-, and hexa(ethlyene glycol) groups were 90, 50 and 30 μM, respectively. No significant inhibition of attachment to the monolayers was observed when control peptide GRGES or scrambled peptide GRDGS were included in the medium, indicating the specificity of the cell–substrate interaction.

3.5. Microenvironment of ligand influences formation of stress fibers and focal contacts

We next used double-label immunofluorescence microscopy to determine whether substrate-dependent differences in cellular morphology were accompanied by differences in stress fibers and focal contacts. A series of monolayers presenting 0.5% GRGDS among oligo(ethylene glycol groups) was used because the morphologies of cells attached to these substrates ranged from well-spread [on tri(ethylene glycol)] to rounded [on hexa(ethylene glycol)]. The optical micrographs in Fig. 7A and B depict a well-spread cell on a monolayer presenting 0.5% GRGDS and tri(ethylene glycol) groups. Inspection of these micrographs shows that actin stress fibers (Fig. 7A) colocalize with focal adhesion complexes staining positive for vinculin (Fig. 7B). These structures resemble those found in cells attached to a glass coverslip coated with a 10 μg/ml solution of fibronectin (Fig. 7C and D). Cells attached to monolayers presenting 1% peptide among each oligo(ethylene glycol) group showed similar patterns of staining (data not shown). Fig. 7E and F show the distribution of *f*-actin and vinculin in a rounded cell attached to a monolayer presenting the 0.5% GRGDS peptide among hexa(ethylene glycol) groups. This cell contains fewer and less-defined focal adhesions. The actin filaments in this cell are also shorter than those in cells attached to fibronectin or to monolayers presenting the peptide among tri(ethylene glycol) groups.

4. Discussion

4.1. Both density and microenvironment of ligand influence cell adhesion

In this report, we demonstrate that the integrin-mediated adhesion of Swiss 3T3 fibroblasts depends not only on the density of immobilized ligands, but also on

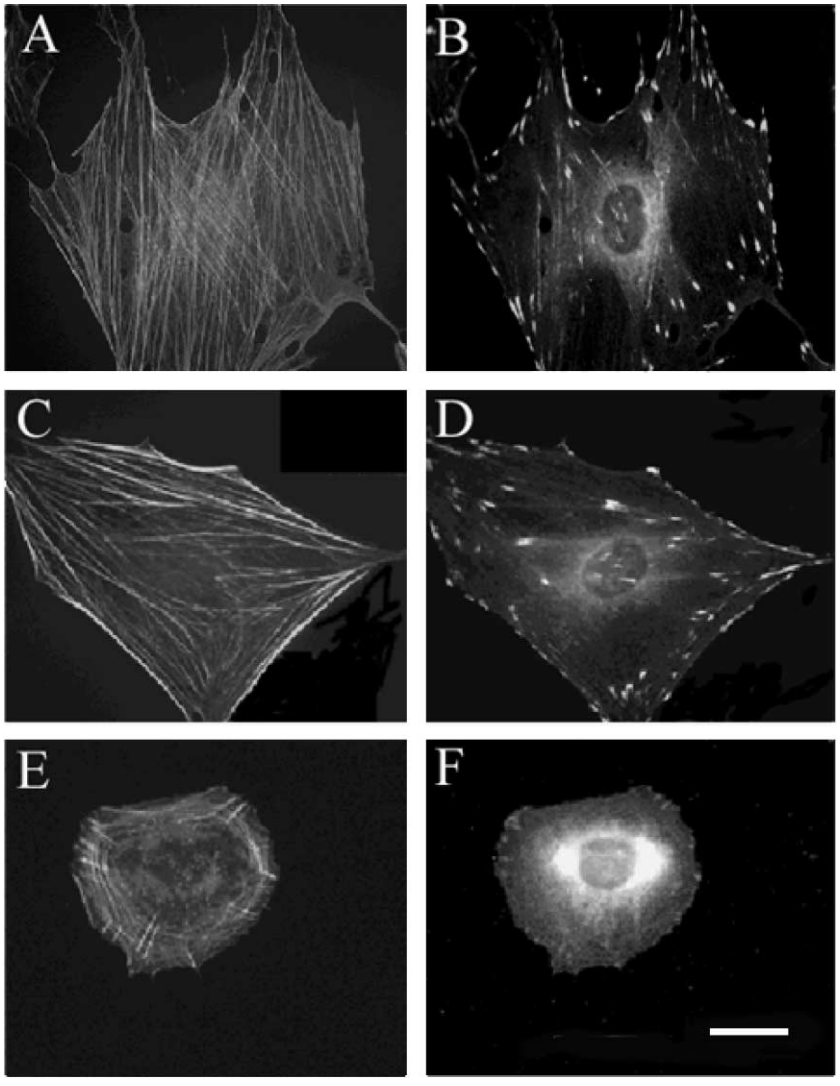

Fig. 7. Fluorescence micrographs of actin filaments and focal adhesions in cells attached to monolayers. Cells attached to monolayers presenting 0.5% GRGDS among tri(ethylene glycol) groups (A and B), hexadecanethiolate coated with fibronectin (C and D), and 0.5% GRGDS among hexa(ethylene glycol) groups (E and F) were stained with Alexa 564-conjugated phalloidin (A, C, and E) and an Alexa 488-conjugated secondary antibody to anti-vinculin IgG (B, D and F). The scale bar represents 15 μm.

the groups surrounding those ligands. Many previous studies that used model substrates to study cell adhesion correlated biological function with density of immobilized ligand. Danilov and Juliano, for example, compared the attachment of Chinese hamster ovary cells to substrates coated with fibronectin and RGD-albumin conjugates [29]. They found that the albumin conjugate supported maximal cell attachment at a coating density of 400 fmol/cm^2, while substrates coated with fibronectin supported maximal attachment at a density of 110 fmol/cm^2. It is difficult, however, to make direct comparisons of cell attachment to these substrates because the structures of the adsorbed protein are likely different for each case and are generally intractable. The presence of more than one cell adhesion motif in proteins like fibronectin further complicates the interpretation of these data [41,42]. These factors suggest that the biological activity of the substrates depends not on the total density of adhesion ligands, but on the fraction of ligands in the protein film that are available for binding and on the microenvironment of those ligands.

The results presented in this paper demonstrate the extent to which the *microenvironment* of immobilized peptide ligands influences cell adhesion to self-assembled monolayers. Increasing the length of the oligo(ethylene glycol) unit on self-assembled monolayers presenting a constant density of GRGDS peptide decreased the attachment and spreading of Swiss 3T3 fibroblasts (Fig. 5). These decreases were not due to insufficient ligand on the monolayer because the density of immobilized ligand on all substrates was well above the thresholds previously shown to support cell spreading and

focal contact formation [11,21–23]. We also ruled out the possibility that protein synthesis during the assay influenced the efficiency of adhesion, since cells treated with cycloheximide gave results similar to those of untreated cells. It is important to note that the assay used in this study is less reproducible and quantitative than radial-flow [43] or centrifuge-based assays [44]. Nevertheless, the observed trends in cell attachment and spreading were both statistically significant and reproducible.

It is likely that differences in cell adhesion to substrates presenting different oligo(ethylene glycol) groups reflect changes in the affinity of integrins for the immobilized peptide. In previous work, relative differences in the free energy of integrin–ligand binding among a series of substrates were correlated with the concentration of soluble peptide required for half-maximal inhibition of cell attachment [27–29,45–48]. We used this assay to inhibit cell attachment to monolayers presenting 1% peptide among a series of glycol groups and found that increasing the length of the glycol group did indeed produce a decrease in the concentration of soluble peptide required to inhibit half-maximal cell attachment (Fig. 6). These results suggest that interactions between cellular receptors and the groups surrounding immobilized ligands influence the affinity of the cell–substrate interaction. It is important to recognize, however, that this assay does not measure the binding constant (K_d) for the monovalent integrin–peptide interaction, since the adhesion of a cell to a substrate is a complex process that involves thousands of ligand–receptor interactions and clustering of receptors.

Increasing the length of the glycol group can influence the binding affinity of integrins for immobilized peptides in several ways. First, hexa(ethylene glycol) groups are expected to crowd the peptide ligand more than tri(ethylene glycol) groups since the longer chains extend closer to the RGD motif (Fig. 1). Second, the peptide ligand may become entangled in the surrounding glycol groups. Each of these processes could influence the accessibility and the conformational flexibility of the peptide, decreasing its ability to bind cellular receptors. Third, cellular integrins may interact repulsively with ethylene glycol groups adjacent to the peptide as the protein approaches the immobilized ligand [49,50]. If the glycol groups extend closer to the ligand, these interactions will occur to a greater extent. Since all three processes will decrease the association rate constant (k_{on}) for the integrin–peptide interaction, our experiments cannot distinguish between them. In fact, it is likely that more than one process occurs in this model system. The length of the glycol group will probably influence the dissociation rate constant (k_{off}) to a smaller extent, since this rate constant is determined largely by interactions within the receptor binding pocket. We are currently using surface plasmon resonance spectroscopy to investigate directly the influence of microenvironment of ligand on the monovalent binding of soluble integrins to immobilized peptides.

The results described above are consistent with data from a recent study that examined cell adhesion to supported bilayers presenting mixtures of peptide amphiphiles and poly(ethylene glycol) lipids [25]. By increasing the length of the poly(ethylene glycol) head group in the film, Tirrell and coworkers altered the accessibility of the peptide to cellular receptors. This work provides an example of how the microenvironment of ligands can be used to influence cell–substrate interactions, but a direct comparison of this study with our work is difficult because the peptide amphiphiles in the bilayer were presented at a much higher density and were free to diffuse laterally.

4.2. The role of polyvalent interactions in cell adhesion

The degree to which the density of immobilized peptide influences cell attachment depends on the length of the oligo(ethylene glycol) group (Fig. 5A). A decrease in the density of immobilized peptide from 1 to 0.1% had a marginal effect on the number of cells that attach to monolayers presenting tri(ethylene glycol) groups, but the same decrease in the density of peptide resulted in an almost complete loss of cell attachment to monolayers presenting hexa(ethylene glycol) groups. These data are consistent with a probabilistic model of cell attachment derived by Lauffenburger and coworkers [51–53]. This model defines the probability that a collision of a cell with a substrate results in attachment as a function of the binding constant of cellular receptors to immobilized ligand and the rate of diffusion of receptors in the plasma membrane. The model assumes that the attachment of a cell to a substrate is kinetically controlled and predicts that (1) the total number of receptors required for adhesion is proportional to the association rate constant (k_{on}) and (2) that the probability of attachment is controlled by the rate of receptor–ligand complex formation during the encounter.

In our experiments, the number of cells (and, to a first approximation, the number of receptors) was constant on all substrates. If increasing the length of the glycol group decreases the integrin–peptide association rate constant, the probability of cell attachment should also decrease. This prediction is consistent with the observed decreases in cell attachment to substrates presenting the peptide among longer glycol groups. The model also suggests that the attachment to monolayers presenting longer glycol groups will be dependent on density: if fewer ligands are available, the probability that a stable adhesion can form will decrease. It is notable that cell attachment to substrates presenting tri(ethylene glycol) groups was not dependent on the density of ligand. One possible explanation for these data is that the sticking probability of cells may be close to its limiting value at all

densities of peptide on substrates that present tri(ethylene glycol).

Cell spreading also showed a greater dependence on density of peptide in the presence of longer glycol groups (Fig. 5B). Unlike cell attachment, which is largely determined by the kinetics of the cell–substrate interaction (k_{on}), cell spreading reflects a dynamic equilibrium between the cell and its environment. Since the degree of cell spreading is determined (at least in part) by a balance between the force generated by cell–substrate interactions and the tension present in the cytoskeleton, any interpretation of trends in cell spreading must take into account the polyvalency of interactions between the cell and the substrate.

Studies using well-defined model systems have shown that polyvalent ligand–receptor interactions often display large binding enhancements over their monovalent counterparts [54–56]. These studies have also revealed that weak monovalent ligands show larger binding enhancements in a polyvalent context than do strong monovalent ligands. These enhancements have been defined most clearly as the ratio of the polyvalent association constant to the monovalent association rate constant (K^{poly}/K^{mono}) [57]. It is difficult to determine this ratio for the adhesion of a cell to a substrate because changes in the free energy of polyvalent binding are far too large to be measured. Nevertheless, the large dependence of cell spreading on density of peptide in Fig. 5B suggests that the ratio K^{poly}/K^{mono} is greater for monolayers that present longer glycol groups. This trend — wherein a collection of weak, monomeric interactions exhibits a large binding enhancement — is an important principle in the adhesion of cells to ECM and to other cells [58,59].

4.3. Comparison of monolayers and protein-coated substrates

The model substrates described here are different in several respects from the protein-coated substrates commonly used for studies of integrin-mediated cell adhesion. First, the interactions between cellular receptors and immobilized ligands are defined completely because the ligands are presented on a surface that is otherwise inert to protein adsorption. This characteristic was verified by the observation that the attachment of cells to the monolayers is completely inhibited by low concentrations of soluble peptide (Fig. 6). Analogous experiments with protein-coated substrates required concentrations of peptide as high as 3 mM to inhibit attachment, and even then 10–20% of cells still remained attached [27–29,45–48]. The presence of additional cell adhesion ligands on protein-coated substrates may have contributed to the difference, but it is also likely that nonspecific adsorption of cell surface proteins to the substrate played a role.

Cells that were well-spread on the monolayers formed actin stress fibers and vinculin-containing focal contacts that were similar to those found in cells attached to fibronectin-coated substrates (Fig. 7). Although it is unreasonable to expect RGD to reproduce all the functions of fibronectin, these results show that the immobilized peptide supports the clustering of integrins and the formation of actin cables. At low densities of peptide, however, the formation of these structures was strongly influenced by the groups surrounding the ligand, since cells attached to substrates presenting the same density of peptide among hexa(ethylene glycol) groups exhibited fewer stress fibers and focal contacts. Clearly, a threshold density of ligand is not the only requirement for the formation of these structures. Since the assembly of stress fibers and focal contacts involve adhesion-mediated signal transduction, it is possible that the groups surrounding peptide ligands may also influence other cellular processes, including apoptosis and differentiation [1–5,16].

5. Conclusions

Subtle changes in microenvironment of ligands in matrix proteins often produce marked changes in biological response. Boettinger and coworkers, for example, showed that collagen or tissue culture polystyrene coated with fibronectin supported the differentiation of myocytes, while bacterial polystyrene presenting the same density of fibronectin (based on radiolabeling) did not [16]. These differences in biological activity were accompanied by differences in antibody binding to the substrates, suggesting that the conformation of the protein — and the microenvironment of ligands in the protein — were substrate-dependent. In vivo, cells may alter the microenvironment of their extracellular ligands by the deposition or degradation of protein [6,60], by mechanical deformation of the matrix [61], or by alteration in the composition or expression of proteoglycans [62].

In this study, we use self-assembled monolayers to examine how the microenvironment of immobilized peptides influences cell attachment and spreading. These substrates have several characteristics that make them particularly well suited for such studies. First, the structure of these monolayers permits strict control over the density and environment of ligands presented at the interface, while synthetic organic chemistry allows the structure of ligands to be controlled and modified. Another important characteristic of these monolayers is that they resist both non-specific adsorption of protein and the remodelling of matrix by attached cultures [12,32–34]. Finally, these substrates can be patterned using microcontact printing to define the shapes, sizes, and positions of cells on a monolayer [63–66]. The monolayers are also compatible with electrochemical

strategies that can modulate, in real time, the presentation of ligands to an attached cell [67,68]. The last characteristic will be particularly important for modeling dynamic changes in the cell–substrate interaction.

This study clearly shows that the density and the microenvironment of immobilized peptide ligands can independently influence cell attachment and spreading. This work also shows that large, density-dependent enhancements of cell attachment and spreading occur when the cell–substrate interaction is weak. These findings will be important for studies that correlate biological function with composition of ligands on a substrate, as they demonstrate how the *presentation* of adhesion motifs on a substrate modify the biological response of mammalian cells. These findings will also be useful in the design of biomaterials that enhance or inhibit specific ligand-receptor interactions [8,9,44,69]. We believe that self-assembled monolayers will be important not only for mechanistic studies for cell adhesion, but also for mechanistic studies of other phenomena that rely on cell-substrate interactions, including signal transduction, cell–cell communication and cell migration.

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