Cell adhesion and motility depend on nanoscale RGD clustering

Gargi Maheshwari¹, Gillian Brown², Douglas A. Lauffenburger¹, Alan Wells³ and Linda G. Griffith¹,*

¹Division of Bioengineering and Environmental Health, Department of Chemical Engineering, and Center for Biomedical Engineering and ²Department of Material Science and Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
³Department of Pathology, University of Pittsburgh and Pittsburgh VAMC, Pittsburgh, PA 15216, USA

*Author for correspondence (e-mail: griff@mit.edu)

Accepted 8 March; published on WWW 18 April 2000

SUMMARY

Integrin adhesion receptors play a crucial role in regulating interactions between cells and extracellular matrix (ECM). Integrin activation initiates multiple intracellular signaling pathways and results in regulation of cell functions such as motility, proliferation and differentiation. Two key observations regarding the biophysical nature of integrin-mediated cell-matrix interactions motivated the present study: (1) cell motility can be regulated by modulating the magnitude of cell-substratum adhesion, by varying cell integrin expression level, integrin-ECM binding affinity or substratum ECM surface density; and (2) integrin clustering enables assembly of multiple cytoplasmic regulatory and structural proteins at sites of aggregated integrin cytoplasmic domains, activating certain intracellular signalling pathways. Here, using a minimal integrin adhesion ligand, YGRGD, we test the hypothesis that ligand clustering can affect cell migration in a manner related to its modulation of cell-substratum adhesion. We employ a synthetic polymer-linking method, which allows us to independently and systematically vary both the average surface density and the local (approx. 50 nm scale) spatial distribution of the YGRGD peptide, against a background otherwise inert with respect to cell adhesion. In this system, the ligand was presented in three alternative spatial distributions: singly, in clusters with an average of five ligands per cluster, or in clusters with an average of nine ligands per cluster; for each of these spatial distributions, a range of average ligand densities (1,000-200,000 ligands/μm²) were examined. Cluster spacing was adjusted in order to present equivalent average ligand densities independently of cluster size. The murine NR6 fibroblast cell line was used as a model because its migration behavior on ECM in the presence and absence of growth factors has been well-characterized and it expresses integrins known to interact with the YGRGD peptide. Using time-lapse videomicroscopy and analysis of individual cell movement paths, we find that NR6 cells can migrate on substrata where adhesion is mediated solely by the YGRGD peptide. As previously observed for migration of NR6 cells on fibronectin, migration speed on YGRGD is a function of the average surface ligand density. Strikingly, clustering of ligand significantly reduced the average ligand density required to support cell migration. In fact, non-clustered integrin ligands support cell attachment but neither full spreading nor haptokinetic or chemokinetic motility. In addition, by quantifying the strength of cell-substratum adhesion, we find that the variation of cell speed with spatial presentation of YGRGD is mediated via its effect on cell adhesion. These effects on motility and adhesion are also observed in the presence of epidermal growth factor (EGF), a known motility-regulating growth factor. Variation in YGRGD presentation also affects the organization of actin filaments within the cell, with a greater number of cells exhibiting stress fibers at higher cluster sizes of YGRGD. Our observations demonstrate that cell motility may be regulated by varying ligand spatial presentation at the nanoscale level, and suggest that integrin clustering is required to support cell locomotion.

Key words: Integrin, RGD, Cell motility, Cell/substratum adhesion

INTRODUCTION

Cell motility across a substratum is strongly affected by the biophysical nature of adhesive interactions (Lauffenburger and Horwitz, 1996). It has been shown both theoretically and experimentally that the average speed of cell locomotion typically exhibits a biphasic dependence on the strength of cell substratum adhesion for a number of cell types, with a maximal value for cell migration speed occurring at an intermediate value of cell-substrate adhesive strength (DiMilla et al., 1991, 1993a,b; Goodman et al., 1989; Huttenlocher et al., 1996; Maheshwari et al., 1999; Palecek et al., 1997; Wu et al., 1994). Systematic variation in cell-substratum adhesion strength has most commonly been achieved by variation of the surface density of ECM proteins (Maheshwari et al., 1999) or anti-integrin antibodies adsorbed onto the substrate (Duband et al., 1991), thus changing the number or strength of bonds between adhesion receptors and the extracellular components. Comparable effects have been achieved in a three-dimensional system by adding systematically varied amounts of fibronectin...
to collagen gels (Kuntz and Saltzman, 1997). Cell-substrate adhesion strength can also be altered, although not yet systematically, by addition of soluble growth factors (Maheshwari et al., 1999).

Accumulating data support the idea that both occupancy and clustering of integrins are required to elicit full cellular responses mediated by integrins (Dedhar and Hannigan, 1996; Ginsberg et al., 1992; Hato et al., 1998; Kornberg et al., 1991; Miyamoto et al., 1995a,b). Integrins show an enhanced propensity to cluster and interact with the cytoskeleton upon ligand binding (LaFlamme et al., 1992; Yauch et al., 1997). Both ligand binding and receptor clustering are needed to engage interactions with a full complement of cytoskeletal and signaling proteins (Miyamoto et al., 1995a,b). Mutations in the cytoplasmic domain that impair integrin diffusion and clustering also impair cell adhesion and spreading (Yauch et al., 1997), processes necessary for migration. The role of integrin aggregation state in cell migration has not been studied explicitly. Activation of focal adhesion kinase, which is associated with integrin clustering, is required for migration (Sieg et al., 1999). At the same time, the presence of focal adhesion complexes inhibits motility and dissolution of focal adhesions is associated with enhanced motility (Angers-Loustau et al., 1999; Burridge and Chrzanowska-Wodnicka, 1996; Dunlevy and Couchman, 1993; Pelham and Wang, 1997; Volk et al., 1984; Xie et al., 1998). Regulation of the state of integrin aggregation thus appears to be involved in regulation of cell motility in a complicated manner.

Many ECM adhesion components are multimeric proteins presenting multiple sites for cell interactions with many different types of cell surface receptors, and would thus be expected to promote clustering of integrins and perhaps of non-integrin adhesion receptors such as syndecans as well. In consideration of this, we developed an entirely new synthetic substrate to present a minimal integrin-binding peptide sequence in a spatially controlled manner against a background inert to protein adsorption and cell adhesion. This novel approach addresses both the specific contributions of integrin ligand and clustering to the process of cell migration, while eliminating other types of receptor-mediated adhesive interactions. The nature of the PEO hydrogel substrate used to present the adhesion ligand is such that cells do not adhere to the substrate even when the gel is immersed first in ECM protein solution for extended times, and thus we also avoid problems arising from adhesion engendered by endogenously secreted ligands. Further, given that both the concentration of integrin ligand as well as its spatial arrangement (through the ability of the ligand to induce integrin clustering) potentially influence observed behaviors, we designed the new system to allow independent variation of each parameter (average ligand surface density and ligand cluster size) over physiologically relevant ranges in order to assess the relative contributions of these two quantitative effects.

We use the monomeric RGD (arg-gly-asp) peptide motif derived from the cell-binding domain of fibronectin as the integrin ligand. The RGD sequence has been shown to interact with αb3 and αb1 integrins (Ruoslahti, 1996), which are present on the EGFR-transduced NR6 fibroblasts that we have previously used to study migration on fibronectin (Maheshwari et al., 1999). In order to achieve interactions solely between the cell and the ligand of choice – i.e. to prevent effects due to serum proteins or cell-secreted proteins – we used a polyethylene oxide (PEO)-based hydrogel as an inert (i.e. non-cell-adhesive) background surface (Griffith and Lopina, 1998). The ligand YGRGD was covalently tethered to the inert background using PEO molecules in a star configuration, enabling nanoscale patterning of the ligand into discrete clusters of 1-9 ligands per cluster with defined spacings of 6-300 nm between clusters (Brown, 1999). We used the minimal sequence YGRGD. RGD ligands that lack additional residues at the C terminus have been reported to be inactive in cell adhesion assays (Hirano et al., 1993; Ruoslahti, 1996). Thus, this minimal low-affinity YGRGD ligand perhaps provides a particularly sensitive probe into the possible effects of ligand clustering on cell response.

We reason that presentation of an integrin ligand in a clustered format may result in more efficient clustering of the ligand-bound integrins compared to the same surface density of ligand presented individually, thereby affecting cellular processes such as motility. We have previously shown that such control of the nanoscale distribution of ligand controls adhesion and spreading of hepatocytes on substrates presenting monomeric, dimeric or trimeric arrangements of carbohydrate ligands for the multimeric hepatic asialoglycoprotein receptor (Griffith and Lopina, 1998). Here, we focus on the regime of individual RGD ligands and small clusters (<10 ligands per cluster, approx. 0.05 μm) because integrin aggregates on the length scale of focal contacts (approx. 1 μm) are associated with inhibition of motility (Pelham and Wang, 1997; Volk et al., 1984).

MATERIALS AND METHODS

Cells and cell culture

The generation of WT NR6 cells, a 3T3-derived murine fibroblastoid cell line lacking endogenous EGF receptor (EGFR) transfected with wild-type human EGFR, has been described previously (Chen et al., 1994). NR6 fibroblasts used in these experiments were from the same stocks as those used previously to characterize migration on fibronectin (Maheshwari et al., 1999). FACS analysis demonstrated that these cells express αaβ1 and αaβ3 integrins (Maheshwari et al., 1999). Both these integrins have been shown to bind RGD adhesion ligands (Aota et al., 1994; Chen et al., 1996). WT NR6 cells were cultured in MEM-α medium supplemented with fetal bovine serum (FBS: 7.5%), penicillin (100 i.u./ml), streptomycin (200 μg/ml), non-essential amino acids (1 mM), sodium pyruvate (1 mM), glutamine (2 mM) and G418 (350 μg/ml). Cells were passaged at subconfluence by trypsinization (0.25%, 1 mM EDTA) and incubated at 37°C, 90% humidity and 5% CO2. Assay medium for performing the migration and adhesion measurements contained MEM-α with 25 mM Hepes, 1 g/l BSA, 1% dialyzed FBS, penicillin (100 i.u./ml), streptomycin (200 μg/ml), non-essential amino acids (1 mM), sodium pyruvate (1 mM), glutamine (2 mM) and G418 (350 μg/ml).

Clustered ligand surfaces

The YGRGD adhesion ligand was presented via polyethylene oxide (PEO) tether against an inert, non-cell-adhesive background (Brown, 1999). We first formed a thin (approx. 300 nm) radiation-crosslinked PEO hydrogel on PEO-silane treated glass coverslips to create a substrate that completely inhibited WT NR6 cell adhesion so that cell interactions with the final ligand-modified substrate could be attributed solely to RGD-receptor interactions.
The YGRGD peptide was attached to the PEG hydrogel-modified coverslips using star PEO tethers in an approach that allows the average surface density and the local (scale of approx. 50 nm) spatial distribution of RGD peptide to be controlled independently. Star PEO comprises many PEO arms emanating from a central core. They have been synthesized with functionalities n = 10-100 and M_w=3,000-10,000 (Gnanou et al., 1988). Thus, by linking 1...n RGD peptides per star, blocking remaining unreacted star arms, and subsequently diluting RGD-modified stars with defined proportions of blank (no RGD modification) PEO stars before grating them to the substrate, RGD peptide may be presented either homogeneously (i.e. one RGD ligand per star) or in discrete clusters (i.e. multiple RGD ligands per star) against an inert PEO background (Fig. 1). In short, protected 125I-YGRGD peptide (Tyr-Gly-Arg(phenyl)-Gly-Asp(bu), American Peptide), was covalently conjugated to star PEO (fave=35, M_w=9100, Shearwater Polymers) and then covalently linked to the PEO hydrogel substrates using standard sulfoNHS/EDC crosslinking chemistries (Brown, 1999). The number of YGRGD molecules per star was controlled via the reaction stoichiometry to achieve stars with an average of 1, 5 or 9 ligands per star molecule to provide a range of cluster sizes.

To generate surfaces as shown in Fig. 1, star PEO-RGD conjugates were covalently linked to the PEO hydrogel substrates from solutions containing a mixture of these conjugates and blank stars in defined proportions. Unreacted chain ends were then blocked with Tris-HCl. The peptide was depoected before cell culture experiments. By varying the proportion of RGD-star PEO to blank star PEO from 1% to 100% in the solution used to prepare the substrates, average ligand densities ranging from 1,000-200,000 RGD molecules/μm² were achieved, with properties shown in Table 1. The average center-to-center distance between the clusters was 6-300 nm. A panel of substrates comprising five different average ligand densities for each of the three cluster sizes was prepared with the aim of achieving comparable ligand densities for each of the three cluster sizes. We confirmed that the resulting densities elicited a physiological range of cell behaviors where the chemistry was not limiting.

After final preparation, coverslips were glued to the bottom of 24-well plates using an optically clear adhesive (Norland Chemicals, New Brunswick, NJ, USA).

**Fibronectin substrata**

Data for cell adhesion, migration and cytoskeletal organization on fibronectin-coated substrata in the presence and absence of EGF were reported previously (Maheshwari et al., 1999) and are included here where appropriate for comparison. To prepare fibronectin-coated substrata, glass coverslips were silanized, glued to the bottom of either 35 mm polystyrene Petri dishes (for migration studies) or 24-well plates (for adhesion studies), and coated with fibronectin by incubation with 0.2 ml fibronectin solution/cm² surface area of substrate for 2 hours. Substrates were then incubated with a 1% solution of bovine serum albumin for 1 hour to block further non-specific adsorption of protein. Fibronectin concentration in the coating solution was varied from 0.1-3 μg/ml. The amount of fibronectin associated with the substrate for each coating concentration was determined using radiolabelled fibronectin according to the methods previously described (Asthagiri et al., 1999). Surface molecular densities of fibronectin were 26, 63, 180 and 420 molecules/μm² for coating concentrations of 0.1, 0.3, 1 and 3 μg/ml, respectively. Molecular densities of ligand reported in the results were calculated by assuming that each fibronectin molecule offered one integrin binding site.

**Migration assay**

Cell migration speed was measured using time-lapse videomicroscopy of single cells (Maheshwari et al., 1999). 30,000 cells were plated onto 35 mm dishes in 2.5 ml serum-free medium. The medium was changed 12 hours post-seeding to 2.5 ml assay medium with or without 25 nM EGF and incubated at 37°C in humidified air for 8 hours. At this cell density, soluble ligand concentration is relatively unchanged over a 24 hour period (Reddy et al., 1996). Mineral oil (3 ml) was added to the dish to prevent evaporation, and the dish was then placed in a heated stage insert for a Luidl 995008 motorized stage on a Zeiss Axiovert 35 microscope. Cell boundaries and centroids were identified using image processing software developed by Engineering Technology Center (Mystic, CT, USA) running under a LabVIEW (National Instruments, Austin TX, USA) and Concept Vi (Mystic, CT, USA) environment. 5-10 cells per field in 10 different fields were scanned every 15 minutes for up to 20 hours. The x and y coordinates of the cell centroids were recorded every 15 minutes. Single cell speed is calculated by determining the total path length as measured by the total centroid displacement divided by the tracking time. The reported cell speed ± s.e.m. for each condition is an average over 70-100 cells. For purposes of testing transient effects, cell speeds were calculated every 15 minutes by quantifying the centroid displacement every 15 minutes over a 20-hour period starting immediately after addition of EGF, or in the control case, immediately after the medium was changed to the assay medium. Under such conditions, cell speed increases toward a plateau for 6-8 hours following addition of EGF (Maheshwari et al., 1999). Hence all subsequent migration measurements were carried out following an 8-hour incubation period.

**Adhesion assay**

A centrifugal cell detachment assay was performed as previously described (Chu et al., 1994) to assess cell-substrate adhesion strength. Briefly, substrates were glued to the bottom of 24-well plates and were plated with 20,000 cells per well in serum-free conditions for 12 hours. The medium was then changed to the assay medium with or without 25 nM EGF. The wells were filled with medium and sealed using sealing tape to avoid air bubbles. The plates were then inverted and spun in a swing bucket SH-3000 rotor in a bench top Sorvall centrifuge for 10 minutes at 25°C at 800 g. During each experiment, one plate at the highest cluster size and highest average surface YGRGD density without EGF was kept at 1 g and used as a control. Cell number was quantified by manually counting cells in a defined well area. At least four wells were used at each condition and four fields were counted per well with each field in the control containing 300-400 cells. The cell number per well was normalized to the average cell number in the control well to obtain fraction adherent cells for each condition.

In previous work characterizing cell migration on fibronectin, we measured centrifugal detachment of cells at spun at 400, 600 and 800 g and used standard procedures to calculate the mean force on the

**Table 1. Quantitative properties of ligand presentation cases**

<table>
<thead>
<tr>
<th>n_c</th>
<th>Average RGD density (molecules/μm²)</th>
<th>Average cluster spacing (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>900</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>2600</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>6000</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>30,000</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>6800</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>30,000</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>200,000</td>
<td>11</td>
</tr>
<tr>
<td>9</td>
<td>900</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>2300</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>6200</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>123,000</td>
<td>26</td>
</tr>
</tbody>
</table>
cells to generate a plot of fraction of adherent cells versus centrifugal force, enabling calculation of the force required to detach 50% of the cells (F_{50}) as a comparative measure of cell adhesion strength (Maheshwari et al., 1999). Measurement of detachment at three different centrifugal force values would require a prohibitively large number of YGRGD substrates. We thus used the single force which would give us the most discriminatory range among substrates (i.e. where fraction-adherent cells did not saturate out at 0 or 1), and used fraction of adherent cells at that force as the basis of comparison.

Cytoskeletal staining
For actin filament and vinculin visualization, cells were rinsed with PBS and fixed using 3.7% paraformaldehyde. The cells were then rinsed with PBS and permeabilized by incubation for 3 minutes in 0.1% Triton X-100 (Sigma Chemical Co.). After rinsing twice in PBS, the cells were incubated for 45 minutes at room temperature in a humidified chamber with a 1:100 dilution of monoclonal mouse anti vinculin antibody (Sigma Chemical Co.). The samples were then rinsed with PBS and incubated in a mixture of rhodamine-phalloidin (1:200 dilution, Sigma Chemical Co.) and Alexa 488-conjugated goat anti-mouse secondary antibody (1:50, Molecular Probes, OR, USA) in PBS for 45 minutes in a dark humidified chamber. The samples were then thoroughly washed and mounted on glass slides using Prolong Antifade solution (Molecular Probes, OR, USA). The preparations were then visualized on a Zeiss 35 inverted fluorescence microscope and images captured using a Hamamatsu cooled CCD camera on OpenLab imaging software. For the fibronectin controls, cells were plated on coverslips coated with 1 mg/ml fibronectin coating concentration and blocked with 1% BSA in PBS as described previously (Maheshwari et al., 1999). Approximately 250 cells were scored from four coverslips to quantify the proportion of cells with well-formed actin stress fibers at each condition.

RESULTS

Cell adhesion and spreading on polymer-tethered RGD is RGD-specific
To assess the specificity of cell-substrate interactions, cell adhesion to the plain and RGD-modified substrates was investigated by seeding wild-type NR6 fibroblasts in medium containing 7.5% FBS and maintained for up to 2 days. No cell adhesion was observed on plain substrates, substrates modified with stars lacking RGD, or substrates that were modified with stars prepared with unprotected RGD peptide. These substrates were also non-adhesive when first soaked in 10 μg/ml fibronectin solution for 24 hours before cell seeding. Adhesion on all RGD-modified substrates was completely inhibited by 2 mM soluble RGD peptide (GRGDSP). Cell morphology depended on the average RGD density, with cells spreading maximally on the substrates presenting >1 RGD/star and >2000 RGD/μm². Addition of RGD but not nonsense peptide (2 mM) to the cells 6 hours after seeding caused cell rounding, followed by respreading after changing to peptide-free medium. Thus, the substrates appear to interact with NR6 fibroblasts solely via the YGRGD ligand.

Cell speed depends on average surface density of RGD and on the presence of EGF
Surfaces with systematically varied average ligand density (600-200,000 YGRGD molecules/μm²) and average cluster size (n_{cl}=1,5,9) were synthesized and characterized to confirm spatial clustering of the ligands (Brown, 1999). Cell speed was quantified by time lapse videomicroscopy of single cells over a 12 hour time period in the presence and absence of soluble EGF.

We found that the YGRGD peptide is able to support cell
migration in the absence of other cell-substrate adhesive interactions. At each YGRGD cluster size, cell speeds were greater at greater values of average YGRGD surface density. This trend of increasing cell speed with increasing ligand concentration was significantly more pronounced in the presence of EGF. At $n_{cl}=5$ and 9, cell speeds appeared to reach a plateau of approx. 16 $\mu$m/hour in the absence of EGF and approx. 31 $\mu$m/hour in the presence of EGF (Fig. 2).

The trends in NR6 cell migration speed as a function of ligand density show some similarities to the trends observed for NR6 migration on fibronectin (Maheshwari et al., 1999) but there are also some important distinctions, which can be seen most clearly when the data in Fig. 2 are split into separate graphs for ± EGF and shown with the corresponding data for migration on fibronectin derived from Maheshwari et al. (1999) (Fig. 3A,B). In the absence of EGF (Fig. 3A), cell speed on fibronectin is approximately constant and not statistically different from the plateau value for migration on YGRGD. The data for fibronectin show a comparable trend to data for YGRGD where $n_{cl}=9$, but fibronectin data do not extend to low ligand densities, where speeds on YGRGD cluster sizes are below the plateau. Thus, no important differences between NR6 fibroblast migration on YGRGD and fibronectin are discerned from the data in the absence of EGF. In the presence of EGF, however, migration speeds on YGRGD and fibronectin exhibit distinct behaviors. Migration speeds on clustered YGRGD show a monotonic increase to a plateau with increasing ligand density, while speeds on fibronectin increase to a maximum at intermediate ligand densities and return to a value approximately that of the plateau at high ligand density (Fig. 3B). This biphasic behavior is not observed on the YGRGD substrate at any cluster size studied.

**Cell speed depends on the average cluster size of YGRGD**

At a given value of average YGRGD surface density, cell speeds were significantly greater for larger cluster sizes, both in the presence and absence of soluble EGF. For example, at an average YGRGD surface density of $10^4$ molecules/μm$^2$, increasing $n_{cl}$ from 1 to 5 to 9 increased the cell speed from 1.2 to 8.8 to 14.6 mm/hour, respectively, in the absence of EGF, and from 1.9 to 13.9 to 29.7 $\mu$m/hour, respectively, in the presence of EGF. Ligand clustering (i.e. $n_{cl}>1$) was required to

Fig. 3. NR6 cell migration is similar to migration on fibronectin in the absence of EGF but lacks the biphasic behavior observed on fibronectin in the presence of EGF. NR6 cell migration speed as a function of ligand density for migration on synthetic substrates (YGRGD) and fibronectin in the presence of EGF (A) and in the absence of EGF (B). Each fibronectin molecule and each YGRGD is counted as one ligand. Data for fibronectin are from Maheswari et al. (1999).

Fig. 4. Ligand density and spatial organization influences cell-substrate adhesion strength. Fraction of cells remaining adherent to the substrate after inversion and centrifugation at 800 g as a function of the average YGRGD ligand concentration on the surface in the presence (filled symbols, solid lines) and absence (open symbols, dotted lines) of EGF for randomly distributed ligand (squares) or ligand presented in clusters of 5 (triangles) or 9 (circles) YGRGD ligands per cluster. Adhesion was measured 20 hours after initial plating, corresponding to the time that the migration assay was initiated.
reach maximal migration speeds, which were independent of cluster size but dependent on EGF as discussed above. For a given cluster size, the average YGRGD ligand surface density required to elicit maximal migration speed was comparable in the presence and absence of EGF (Fig. 2). The average ligand density required to elicit maximal migration speed was, however, strikingly dependent on cluster size. The plateau is achieved at around $5,000\text{--}8,000$ YGRGD/$\mu m^2$ for $n_{cl}=9$, around $30,000\text{--}60,000$ YGRGD/$\mu m^2$ for $n_{cl}=5$, and no plateau is discernible for $n_{cl}=1$, where the maximum ligand density achievable by our synthesis method was 30,000 YGRGD/$\mu m^2$ (Fig. 2).

These results indicate that adhesion ligand presentation has a significant effect on both the basal speeds and growth factor stimulated speeds of NR6 fibroblasts. Further, both parameters characterizing ligand presentation, average surface density and local cluster size, exert an influence on cell speed.

**Cell/substratum adhesion strength depends on characteristics of YGRGD ligand presentation**

In earlier theoretical and experimental studies, it has been shown that cell speed may be governed in part by the strength of cell-substratum adhesion (DiMilla et al., 1991, 1993a; Maheshwari et al., 1999; Palecek et al., 1997; Wu et al., 1994). Therefore, in order to determine whether the effect of YGRGD presentation on cell speed is mediated via its effect on cell substratum adhesion, we quantified cell-substratum adhesion using the centrifugation adhesion assay to obtain the fraction of adherent cells after centrifugation at a defined force as a metric of comparison.

As expected, cell adhesion strength was greater at greater ligand densities for each of the cluster sizes (Fig. 4). This trend was also observed in the presence of soluble EGF. However, addition of soluble EGF decreased the strength of cell-substratum adhesion at each surface condition (Fig. 4). This decrease of adhesion in the presence of EGF is consistent with earlier studies on natural ECM molecules such as fibronectin and amgel (Maheshwari et al., 1999; Wells et al., 1998). At $n_{cl}=5$ and 9, the strength of cell-substratum adhesion appears to be approaching a plateau, suggesting that further increase in the average YGRGD surface density may not result in significant increase in the strength of cell-substratum adhesion.

We observe that a lower average surface YGRGD density is required to achieve a particular value of cell-substratum adhesion when YGRGD is presented in a clustered format. For example, in order to have 30% of cells adherent in the presence of EGF following centrifugation, average surface YGRGD densities of 7400 and 62,000 molecules/$\mu m^2$ are required when YGRGD is presented in clusters of $n_{cl}=9$ or 5, respectively, and fewer than 30% of cells remain adherent at all values of ligand density for $n_{cl}=1$. Similarly, in the absence of EGF, 4200 and 50,000 molecules/$\mu m^2$ are required when using clusters of 9 and 5, respectively, to have 15% cells left adherent following centrifugation (Fig. 4), whereas fewer than 15% of the cells remain adherent following centrifugation for all ligand densities (up to 30,000 molecules/$\mu m^2$) for $n_{cl}=1$.

**Actin stress fiber formation depends on characteristics of RGD ligand presentation**

Cell adhesion and migration on YGRGD are dependent on its average surface density and local cluster size. In order to determine whether these parameters that characterize RGD presentation affect the cytoskeletal organization within the cell, actin filaments were visualized in the absence of EGF (Fig. 5A). We chose EGF- conditions because EGF reduces the number of focal adhesions (Xie et al., 1998). Cells were also

![Fig. 5. Spatial arrangement of RGD affects cytoskeletal organization.](image)

(A) Actin stress fibers (left panels) and vinculin stains (right panels) were visualized at varying RGD densities and cluster sizes. Shown here are typical stains from cells plated on surfaces coated with (i) 1 $\mu g/ml$ fibronectin, (ii) 100% RGD-modified star PEO molecules with $n_{cl}=9$ and (iii) 100% RGD-modified star PEO molecules with $n_{cl}=1$. (B) Cells were scored for actin stress fiber formation with varying RGD spatial arrangements. Approximately 140 cells from three independent experiments were scored for each condition.
stained for vinculin, another adhesion component. We found that, in the absence of EGF, a greater fraction of cells exhibited stress fibers at greater values of average YGRGD surface density (Fig. 5B). In addition, for the same average YGRGD density, a significantly higher fraction of cells exhibited well-formed stress fibers and focal contacts when the ligand was presented in a clustered \( n_{cl}=9 \) versus a random individual \( n_{cl}=1 \) format. Punctate characteristic vinculin staining was also almost completely absent on surfaces with \( n_{cl}=1 \), while those with \( n_{cl}=9 \) exhibited significant vinculin stains (Fig. 5A). This suggests that presentation of the YGRGD ligand in a clustered format enables formation of focal contacts in a more effective manner than presentation of the ligand at a uniform density (in the absence of EGF). In light of the recent report on the molecular heterogeneity in both cytoplasmic proteins and integrin type contained in focal adhesions, it is also possible that unclustered ligand results in adhesions devoid of vinculin and enriched in tensin (Zamir et al., 1999). Cells adhered on surfaces with \( n_{cl}=1 \), but these surfaces were unable to support stress fiber formation, suggesting that presentation of RGD in a manner that does not facilitate clustering of integrins also does not support the formation of stress fibers. This lack of stress fibers in the absence of EGF appears to be distinct from the loss of stress fibers one observes in cells stimulated to migrate (Volk et al., 1984), as the cells on \( n_{cl}=1 \) are basically stationary.

**DISCUSSION**

We aimed to illuminate the role of integrin adhesion ligand clustering in regulating cell migration, by comparing cell migration behavior on substrates that present covalently tethered integrin ligand in a spatially distributed individual or clustered fashion over a range of comparable average ligand densities, and by examining the underlying biophysical processes of adhesion that might be affected by resulting integrin occupation. We used surfaces with the ligand YGRGD patterned at the length scale of integrins and ECM molecules (approx. 50 nm); i.e. a length scale significantly smaller than that typically associated with focal adhesions (>500 nm).

Our most striking finding is that the migration speeds of NR6 fibroblasts on YGRGD ligand strongly depend on ligand spatial organization on the substrate, with a clustered presentation enhancing cell migration speeds at each ligand density studied both in the presence and absence of EGF (Fig. 2). Unclustered ligand \( n_{cl}=1 \) barely supported NR6 motility (maximum migration speed approx. 1 \( \mu \)m/hour) at a ligand density of 30,000 RGD/\( \mu \)m\(^2\), the highest density that could be attained for unclustered ligand with the surface-tethering chemistry we used. In contrast, clustered ligand \( n_{cl}=9 \) supported motility (-12 \( \mu \)m/hour without EGF; approx. 20 \( \mu \)m/hour with EGF) at an average ligand density of just 2300 RGD/\( \mu \)m\(^2\), or approx. 250 clusters/\( \mu \)m\(^2\).

Secondly, we observe that migration speeds on clustered YGRGD substrates exhibit similar behavior to migration on intact fibronectin as a function of ligand density in the absence of EGF (Fig. 3A), but show plateau rather than biphasic behavior as a function of ligand density in the presence of EGF (Fig. 3B). One interpretation of the different behavior on YGRGD and fibronectin in the presence of EGF is that additional signaling pathways are activated by cells adhering to fibronectin compared to the minimal fibronectin-derived sequence YGRGD. These signaling pathways may act in concert with EGFR-activated signaling to change cell contractile force, membrane extension activity, or other processes necessary for migration. Both fibronectin and YGRGD engage \( \alpha_5\beta_3 \) and \( \alpha_5\beta_1 \) integrins, but fibronectin can in addition interact with cells via receptors that bind to non-RGD sites in the C-terminal heparin-binding domain (Bloom et al., 1999; Izzard et al., 1986; Woods et al., 1993).

To help interpret these results regarding cell migration on RGD, the data in Figs 3 and 4, along with corresponding data for cell behavior on fibronectin from Maheshwari et al. (1999), were re-analyzed to show cell speed as a function of cell-substratum adhesion (Fig. 6). In this format, all data for both YGRGD and for fibronectin collapse onto two curves, one in the absence of EGF and the other in the presence of EGF. The curve representing results in the presence of EGF is shifted to the left of the curve in the absence of EGF, indicating decreased adhesion obtained in the presence of EGF. We have previously shown that addition of soluble EGF to NR6 fibroblasts results in a sustained decrease in adhesion to fibronectin (Maheshwari et al., 1999). Migration speeds are consequently altered in the presence of EGF, but not in an entirely predictable manner based on correlations between speed and adhesion observed in the absence of EGF. For a given value of adhesion strength, speeds are greater in the presence of EGF (Maheshwari et al.,

![Fig. 6. Relationship between cell-substratum adhesion strength and cell migration speed, reploting the data from Figs 3 and 4 (along with previous data for adhesion strength on fibronectin-coated substrata; Maheshwari et al., 1999). In the case of no EGF, the curves for 1, 5 and 9 ligends per polymer star all show a common relationship. Similarly, for the case of 25 nM EGF, the curves for 1, 5 and 9 ligands per polymer star all show a different common relationship.

---

**Table 1:**

<table>
<thead>
<tr>
<th>Ligand Density</th>
<th>Cell Speed (( \mu )m/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 RGD/star</td>
<td>40</td>
</tr>
<tr>
<td>5 RGD/star</td>
<td>30</td>
</tr>
<tr>
<td>9 RGD/star</td>
<td>20</td>
</tr>
</tbody>
</table>

**Fig. 6.** Relationship between cell-substratum adhesion strength and cell migration speed, reploting the data from Figs 3 and 4 (along with previous data for adhesion strength on fibronectin-coated substrata; Maheshwari et al., 1999). In the case of no EGF, the curves for 1, 5 and 9 ligands per polymer star all show a common relationship. Similarly, for the case of 25 nM EGF, the curves for 1, 5 and 9 ligands per polymer star all show a different common relationship.
These results are consistent with our hypothesis that adhesion strength serves as one primary physical parameter in cell migration, but one which integrates with others including cytoskeletal contractile force, membrane protrusion frequency, and front-rear asymmetry, as described in a detailed biophysical model of migration proposed earlier (DiMilla et al., 1991) and discussed in more detail in our previous study of the biophysical aspects of NR6 fibroblast migration on fibronectin (Maheshwari et al., 1999). Biochemical signaling initiated by growth factors may alter the values of parameters characterizing these processes in ways that are now being illuminated with molecular-level manipulation of specific system components (Bailly et al., 1998; Greenwood and Murphy-Ullrich, 1998; Wells et al., 1998).

Thus, adhesion strength is the primary parameter underlying the observed differences in cell migration behavior on different YGRGD cluster sizes and on YGRGD and fibronectin. Further, EGF acts to decrease cell adhesion and increase cell speed in the same way for cells on fibronectin and on the minimal fibronectin peptide YGRGD, and on YGRGD substrates of different cluster sizes. We cautiously interpret these results as preliminary evidence that cell motility can be regulated by appropriate presentation of simple adhesion sequences that are amenable to incorporation in synthetic materials for tissue engineering.

Although the data for YGRGD fall on the previously obtained curves for fibronectin as shown in Fig. 6, a notable difference in the data for YGRGD is that adhesion strength is restricted to relatively low values compared to fibronectin. The data in Fig. 4 suggest that adhesion strength may reach a plateau value on YGRGD substrates, although data at higher YGRGD ligand densities (not possible in our current system) is needed to confirm this. A plateau in adhesion strength would be expected if the ligand density exceeds that required to bind available integrins at the cell-substrate interface (Palecek et al., 1997). Typical integrin expression is of the order of 100,000 receptors/cell (Akiyama and Yamada, 1985), yielding a receptor density of approx. 100 integrins/μm² for spread cells (cell area was measured at approx. 1500 μm²). The region of the apparent plateau in Fig. 4 corresponds to ligand densities of approx. 200,000 YGRGD/μm², or about 200-fold greater than the approximate receptor density. It is possible, then, that the ligand density is saturating in the upper ranges, although measurement of receptor occupancy would be required to confirm this.

Detachment of cells from the substrate results from breakage of either integrin-cytoskeletal bonds or integrin-matrix bonds. Plateau values of adhesion strength, achieved at maximal receptor occupancy, should in general be strongly governed by the strength of the weaker bond (Saterbak and Lauffenburger, 1996). Among the synthetic fibronectin-derived peptide adhesion ligands, the sequence used in the present studies, Y(Y)GRGD, is of relatively low affinity. This particular peptide has, in fact, been reported as apparently inactive in soluble adhesion-inhibition assays and immobilized adhesion-promotion assays compared to linear peptides possessing at least one additional residue at the carboxyl terminus (Hirano et al., 1993; Ruoslahti, 1996). Extended linear peptides such as GRGDSP exhibit, in turn, integrin-binding affinities approximately 1000-fold lower than intact fibronectin (Ruoslahti, 1996). Because bond adhesion strength diminishes as bond affinity is decreased (Kuo and Lauffenburger, 1993), we believe that the maximum value of cell adhesion strength to the synthetic substrates used in the present study is limited by the very low affinity of this particular integrin-ligand bond. We are currently investigating this using higher affinity peptide ligands. We predict that on surfaces modified with higher affinity ligands, cell speed would be a biphasic function of adhesion strength, identical to that on fibronectin; i.e. that the curves for synthetic substrates shown in Fig. 6 will map along the fibronectin curves both in the presence and absence of EGF. Such a result would be in concordance with the results reported by Palecek et al., where integrin affinity was systematically varied by changing the integrin type and activation state (Palecek et al., 1997). Thus, we would predict that for higher affinity ligands, the curves for speed versus average ligand density will be shifted to the left of those in Fig. 3 and that they will be steeper and biphasic, due to effects described above.

Our observation that ligand cluster size matters, rather than merely clustering versus not, is perhaps more surprising and our interpretation more speculative at present. It is not unexpected that observed cell behaviors (adhesion, migration speed, cytoskeletal organization) are comparable for ncl=5 and ncl=9 at the very highest ligand densities; these substrates are prepared from solutions containing 100% ligand-modified star PEO and thus present an essentially homogeneous field of YGRGD (i.e. no bare patches) at densities far in excess of the receptor density. At lower ligand densities, the surface is >90% bare (inert PEO) with scattered clusters of YGRGD ligand. In our method of surface preparation, the clusters are distributed randomly (rather than uniformly spaced) on the surface, and thus a small fraction of the clusters could be adjacent to another cluster, creating an effectively larger cluster.

One interpretation of our results is that there exists both a minimum threshold cluster size and a minimum number of clusters required in order to achieve significant values of adhesion and migration, although we cannot precisely pinpoint the values of these parameters. In our experimental system, effects are observed for ncl=9 and approx. 250 clusters/μm² (30,000 clusters/cell) and for ncl=5 and approx. 12,000 clusters/μm² (1,200,000 clusters/cell). The size scale of an individual cluster is a few tens of nm, and the average center-to-center spacing between individual clusters at approx. 250 clusters/μm² is 60 nm. Yet, in the absence of EGF to stimulate migration, we see focal adhesions and well-formed stress fibers for ncl=9 and approx. 250 clusters/μm², suggesting a larger aggregation of cytoplasmic proteins and integrins than could be obtained in individual clusters.

The threshold cluster spacings we observe for significant focal adhesion and stress fiber formation in NR6 fibroblasts – approx. 60 nm for ncl=9, 9 nm for ncl=5 and <6 nm for individually presented molecules – are substantially lower (closer) than the previously reported value of 140 nm for focal adhesion and stress fiber formation by fibroblasts on the randomly immobilized ligand GRGDY (Massia and Hubbell, 1991). Several factors potentially contribute to this difference. As noted previously, the YGRGD ligand used here has a much lower affinity than the GRGDY ligand, and thus a much higher ligand density would be required to obtain equivalent values of receptor occupancy. We also use substrates that are far more compliant than the rigid silanized glass used by Massia and
Hubbell; our substrates present a thick (approx. 300 nm) hydrogel, and the PEO star arm tethers act as long spacers as they can extend from their random coil state to a fully extended length of approx. 60 nm. Matrix rigidity enables strengthening of integrin-cytoskeletal linkages (Choquet et al., 1997), and formation of stable focal contacts is facilitated on rigid compared to compliant substrates (Pelham and Wang, 1997), and thus focal contact formation and cytoskeletal assembly may be relatively more difficult on the PEO substrates we employ. Interestingly, on the monovalent adhesion ligand (1=1), the cells spread but do not form stress fibers, nor do they migrate. This lack of stress fiber formation does not imply that other cell behaviors are impaired on compliant substrates; indeed, migration requires focal adhesion turnover (Dunlevy and Couchman, 1993; Xie et al., 1998), and is enhanced on compliant relative to rigid substrates (Pelham and Wang, 1997).

These results extend our previous observations, obtained with hepatocyte adhesion and spreading on carbohydrate ligands (Griffith and Lopina, 1998), that nanoscale pattern of adhesion ligand presentation significantly influences cell function. Application of these types of novel biomaterial surfaces could permit rigorous future studies of the effects of specific protein domains, and rational design of a minimalistic artificial matrix based on simultaneous presentation of different peptide fragments. Additionally, fundamental issues regarding synergies between ligated growth factor receptors and integrins may be addressed using similar synthetic polymer systems. We have previously demonstrated that substrate-tethered EGF remains competent to signal DNA synthesis and induce morphological changes in hepatocytes (Kuhl and Griffith-Cima, 1996). We are currently developing new polymer systems that allow facile tethering of growth factors and integrin ligands either within the same polymer cluster or on separate clusters presented in segregated domains (Banerjee et al., 2000; Walton et al., 1997). These endeavors will enable dissection of the relationships between integrin and growth factor signaling pathways.

This work was partially supported by grants BES96-32714 and BES96-17553 from the NSF Bioengineering and Environmental Systems program to L.G.G. and A.W. and NIH grant GM53905 to D.A.L. G.L.B. was supported by Corning, Inc., and G.M. by a Poitras Fellowship from MIT. Helpful discussions with A. F. Horwitz and R. O. Hynes are gratefully acknowledged. We are also grateful to Lily Koo for her critical reading of the manuscript.

REFERENCES


