Published in final edited form as:

Biomacromolecules. 2008 July ; 9(7): 1843–1851. doi:10.1021/bm8000606.

Integrin-Adhesion Ligand Bond Formation of Preosteoblasts and Stem Cells in Three-Dimensional RGD Presenting Matrices

Susan X. Hsiong^{†,‡}, Nathaniel Huebsch^{‡,§}, Claudia Fischbach^{||}, Hyun Joon Kong[⊥], and David J. Mooney^{*,‡}

Department of Chemical Engineering, University of Michigan, Ann Arbor, MI, School of Engineering and Applied Sciences, Harvard University, Cambridge, MA, Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA, Department of Biomedical Engineering, Cornell University, Ithaca, NY, and Department of Chemical and Biomolecular Engineering, University of Illinois, Urbana, Champaign, IL

Abstract

Cell-interactive polymers have been widely used as synthetic extracellular matrices to regulate cell function and promote tissue regeneration. However, there is a lack of quantitative understanding of the cell–material interface. In this study, integrin-adhesion ligand bond formation of preosteoblasts and D1 stem cells with RGD presenting alginate matrices were examined using FRET and flow cytometry. Bond number increased with adhesion ligand density but did not change with RGD island spacing for both cell types. Integrin expression varied with cell type and substrate in 2D culture, but the integrin expression profiles of both cell types were similar when cultured in 3D RGD presenting substrates and distinct from 2D culture. In summary, combining a FRET technique to quantify bond formation with flow cytometry to elucidate integrin expression can define specific cell–material interactions for a given material system and may be useful for informing biomaterial design strategies for cell-based therapies.

Introduction

Bone tissue engineering offers a promising therapeutic alternative to millions of Americans suffering from bone loss due to trauma, cancer, or congenital defects. Tissue engineering strategies typically involve the transplantation of progenitor stem cells within a synthetic extracellular matrix (ECM) analog to regulate cell function and promote tissue regeneration. However, biomaterial design strategies currently rely on empirical studies to correlate material properties to cell behavior, and there is a lack of quantitative understanding of the cell–matrix interface. In addition, the majority of studies have focused on the effects of matrix cues on differentiated cell types, whereas the stem cell response to these cues is not as well understood.^{1,2} It has been previously reported that matrix cues (e.g., adhesion ligand presentation, substrate stiffness) differentially regulated the growth rate of committed preosteoblasts and stem cells,

*To whom correspondence should be addressed. Address: David J. Mooney Engineering Science Laboratory, Harvard University, SEAS 325 Pierce Hall Cambridge, MA 02138. Phone: 617-384-9624. Fax: 617-496-5264. mooneyd@seas.harvard.edu.

[†]University of Michigan.

[‡]School of Engineering and Applied Sciences, Harvard University.

[§]Harvard-MIT Division of Health Sciences and Technology.

^{||}Cornell University.

[⊥]University of Illinois.

Supporting Information Available. Donor emission intensity of D1 stem cells in 3D RGE-TAMRA-modified alginate gels (DS 2), FRET control (Figure S1). Osteogenic differentiation of D1 stem cells (Figure S2). α_v and α_5 integrin subunit expression of MC3T3 preosteoblasts cultured on RGD-modified alginate substrates (Table S1, DS 8, day 3). This information is available free of charge via the Internet at <http://pubs.acs.org>.

³ and synthetic matrices that promoted bone formation in vivo by transplanted preosteoblasts⁴ failed to induce bone formation by transplanted osteoprogenitor cells without the inclusion of additional signals.⁵ These results demonstrate that the cell response to material cues is dependent on the stage of cell commitment or differentiation, but the mechanism underlying these observed differences is not yet clear. As cell-interactive polymers are often used to deliver stem cells and direct cell fate, it is necessary to obtain a better understanding of how parameters such as bond number and specific integrin–ligand interactions govern the cell response.

The goals of this study were to characterize how specific ECM cues (adhesion ligand density and spacing) correlated with cell–matrix bond number using fluorescence resonance energy transfer (FRET) and whether the stage of cell differentiation and culture conditions influenced integrin receptor expression. Bond formation at the cell–matrix interface was probed using an established FRET technique to quantify cell receptor–adhesion ligand bond number.⁶ FRET is the process by which a donor fluorophore transfers energy to a neighboring acceptor by nonradiative dipole–dipole interaction⁷ and is used to monitor a variety of biological phenomenon, including detection of receptor–ligand binding,⁸ and probing the structure of single molecules.⁹ In this application of FRET, cell–ligand bond formation results in a relative decrease in emission intensity from the donor due to energy transfer from the fluorescein-labeled cells to the acceptor (RGD ligands labeled with TAMRA dye). The relative decrease in donor emission intensity is quantitatively measured and converted to the degree of energy transfer, which is then correlated to bond number using a standard curve as previously described.⁶ The integrin expression profile of MC3T3 preosteoblasts and D1 stem cells was also examined via flow cytometry (FACS) to determine whether differential expression of integrins was dependent on the stage of differentiation and ECM ligand presentation. Specific integrin–ligand interactions govern the cell response to ECM cues, as certain integrin–ligand interactions have been demonstrated to activate differing downstream signaling pathways.¹⁰ ¹¹ The integrins selected for analysis included the α_2 , α_v , α_5 , and β_1 integrins, as these have been reported to be important for ECM-mediated osteoblast differentiation.^{11–13}

The effects of RGD ligand presentation on the integrin expression and number of bonds formed at the cell–matrix interface was examined by varying RGD ligand density and distribution (e.g., spacing) from a model ECM. The ECM used in these studies consisted of a nonadhesive alginate hydrogel modified with synthetic arginine-glycine-aspartic acid (RGD) peptides, which mimic cell-adhesive ligands.¹⁴ The overall adhesion ligand density or total number of RGD peptides per alginate chain, defined as the degree of substitution (DS), is altered by varying the concentration of RGD peptides in the coupling reaction. The nanoscale distribution of adhesion ligands (e.g., RGD island spacing) had been previously shown to influence the proliferation of preosteoblasts and stem cells and was therefore examined in these studies.³ ¹⁵ RGD-modified polymer chains were combined with unmodified polymer chains (DS 0) at various ratios to create adhesive islands (formed by a single alginate chain) in the gels. The nanoscale ligand spacing (termed RGD island spacing) was manipulated by changing the ratio of RGD-coupled and unmodified polymer used to form gels. The DS of the modified polymer chains can be altered in parallel with the island spacing to maintain an overall constant RGD total density in each series of gels.¹⁵ RGD island spacings described were based on values obtained from a two-dimensional (2D) Monte Carlo model that reflected the random packing of polymer chains.¹⁵

The influence of the stage of cell differentiation on bond formation was examined by comparing the number of bonds formed between a clonally derived stem cell line (D1 cells) and cells committed to an osteoblast fate (MC3T3 preosteoblasts). The MC3T3 preosteoblast is a widely utilized model cell-line to study osteoblast differentiation^{16,17} and cell–matrix interactions,¹⁸ and D1 stem cells were selected for these studies as they are homogeneous cell population

similar to the clinically relevant bone marrow stromal cells. The clonally derived D1 bone marrow stromal cell-line (from Balb/c mice) has been demonstrated to differentiate down osteoblast, chondrocyte, and adipocyte lineages^{19–21} and allows a more clear analysis of how matrix cues influence a homogeneous stem cell population.

Experimental Section

Cell Culture

Murine preosteoblast MC3T3-E1 cells, a generous gift from Dr. Renny Franceschi (University of Michigan, Ann Arbor MI), were cultured in α -MEM media (without ascorbic acid, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 100 units/mL penicillin-streptomycin (PS, Invitrogen). A mouse clonally derived bone marrow stromal cell line (D1 cells,²² ATCC, Manassus, VA) was cultured in DMEM media (ATCC) supplemented with 10% fetal bovine serum (ATCC) and 100 units/mL PS. In certain studies, D1 cells were predifferentiated for three weeks in DMEM media (Invitrogen) with 10% fetal bovine serum (Invitrogen) and 100 units/mL PS, supplemented with 50 μ g/mL ascorbic acid and 10 mM β -glycerophosphate (Sigma).

Preparation of Alginate Material

Alginate Substrate for FACS Analysis of Integrins—Peptide-modified alginate was prepared using standard carbodiimide chemistry as described previously¹⁴ from Ultrapure MVG (Pronova, Oslo, Norway) alginate and Gly₄-Arg-Gly-Asp-Ser-Pro (G₄RGDSP) peptides (Peptides International, Inc., Louisville, KY). For analysis of integrins of cells adhered onto 2D alginate substrates, disks (2.54 cm diameter, 1 mm thick) were made using an arch punch (McMaster-Carr, Atlanta, GA) and maintained in phenol red-free α -MEM media with 1% PS overnight until cell seeding. Each disk was placed in a well of a six-well TC plate and maintained in complete phenol red-free α -MEM media for at least 24 h prior to cell seeding. For analysis of integrin expression profile of cells in 3D, cells were encapsulated within alginate disks (10 mm diameter, 1 mm thick).

Alginate Modification for FRET Studies—Peptide-modified alginate was prepared using standard carbodiimide chemistry, as described previously,¹⁴ from Ultrapure MVG (Pronova, Oslo, Norway) alginate and Gly₄-Arg-Gly-Asp-Ala-Ser-Ser-Lys-Tyr (G₄RGDASSKY) and Gly₄-Arg-Gly-Asp-Ala-Ser-Ser-Lys-Tyr-6, carboxytetramethylrhodamine (G₄RGDKY-TAMRA) peptides (Peptides International, Inc., Louisville, KY). The degree of substitution was altered by varying the amount of peptide employed in the reaction in which the peptide is covalently conjugated to the alginate polymer. The reported degrees of substitution are based on previous studies measuring the efficiency of this reaction over a wide range of degree of substitution.¹⁴

FRET Experiments

Cell Culture—Cells were incubated in 5-hexa-decanoaminofluorescein (Invitrogen) containing phenol red-free α -MEM media with 10% fetal bovine serum (Invitrogen) and 100 units/mL PS at a concentration of 0.5 mg/50 mL media per T225 cell culture flask for 24–36 h prior to FRET experiments. After incubation in 5-hexa-decanoaminofluorescein containing media, cells were passaged and resuspended to a concentration of 20 million cells/mL in complete phenol red-free α -MEM media.

A total of 0.5 mL of 2% RGD-modified alginate was mixed via syringe connectors with 0.5 mL of 20×10^6 million cells/mL cell suspension to a final alginate concentration of 1%. The cell-alginate solution was then ionically cross-linked with calcium sulfate slurry at a 25:1 molar ratio and cast between two glass plates. Disks (10 mm diameter, 5 coverslips thick) were

punched out and incubated in complete phenol red-free α -MEM media for 2 h on an orbital shaker at 37 °C and 5% CO₂. The total cell concentration was maintained at 10 million cells/mL for each condition. In certain studies, to investigate the effects of varying peptide DS on the degree of energy transfer, the DS was varied from 1–20. DS is defined as the number of RGD peptides per alginate chain. In studies investigating the effects of RGD island spacing on the degree of energy transfer, the overall peptide density was maintained by mixing RGD-modified alginates with varying DS in different ratios with unmodified alginate.

Measuring Degree of Energy Transfer—After 2 h of incubation at 37°C and 5% CO₂ on an orbital shaker, cell-encapsulated alginate gel samples ($n = 4$ or 5 per condition) were read on a Jorbin Yvon fluorescence spectrometer (Edison, NJ) at an excitation wavelength of 488 nm and emission spectrum was collected from 500 to 650 nm using the Fluoromax 3 software. Each sample was subsequently dissolved in 50 mM EDTA/PBS and incubated at 37 °C for 15 min. Cell counts were obtained using a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA). Fluorescence intensity was normalized to total cell number per sample and expressed as mV/cell. This normalization accounts for variations in cell number in each sample.

The degree of energy transfer was calculated as $1 - F/F_0$, where F is the mV/cell value at 520 nm of the experimental condition (fluorescein labeled cells encapsulated in alginate modified with RGDK-TAMRA peptide) and F_0 is the mV/cell value at 520 nm of the donor control (fluorescein labeled cells encapsulated in alginate modified with RGDK peptide). The number of bonds per cell was calculated using a standard curve that correlated energy transfer to peptide bond formation assessed by using iodinated RGD peptides coupled to alginate polymer chains for MC3T3 and D1 cells, as previously described.⁶ Briefly, cells were mixed with I¹²⁵-G₄RGDASSKY-modified alginate polymer with varying RGD peptide concentrations (N_{RGD}) and the number of RGD peptides bound to cells (N_{bond}) suspended in serum-free media was measured. The degree of energy transfer (D_{FRET}) of fluorescein-labeled cells (donor) encapsulated within rhodamine-G₄RGDASSKY (acceptor) of varying number of RGD peptides (N_{RGD}) was subsequently determined. From these results, the number of bonds formed per cell was correlated to D_{FRET} . The standard curve correlating N_{bond} and D_{FRET} for D1 cells are shown in Supporting Information, Figure S1. The standard curve for MC3T3 has been previously published by Kong et al.⁶

FACS Analysis of Integrins

FACS analysis of the integrin expression profile of cells cultured on tissue culture plastic and RGD-modified alginate hydrogel disks were performed as follows. For tissue culture studies of MC3T3 (passages 10–15) and D1 cells (passages 20–25), cells were grown in T225 tissue culture flasks (BD Falcon) and analyzed while subconfluent. For studies using predifferentiated D1 cells, cells were predifferentiated for 21 days in DMEM media (ATCC) with 10% fetal bovine serum (ATCC) and 100 units/mL PS, supplemented with 50 μ g/mL ascorbic acid and 10 mM β -glycerophosphate (Sigma) from P22–24. For studies of cells grown on RGD-modified alginate hydrogels, cells were seeded onto RGD alginate substrates at a density of 300000 cells per disk and allowed to adhere for 12 h. Each disk was then transferred to a new well of a six-well TC plate and cells were cultured for three days prior to FACS analysis. For 3D studies, cells were encapsulated in alginate hydrogels (2%) at a density of 5 million cells/mL alginate. The cell–alginate solution was then ionically cross-linked with calcium sulfate slurry at a 25:1 molar ratio and cast between two glass plates. After 15 min of gelation, disks (10 mm diameter, 1 mm thick) were punched out and incubated in complete media on an orbital shaker at 37 °C until FACS analysis.

Cells (in tissue culture or seeded onto RGD alginate substrate) were detached by incubating in collagenase I/Dispase digestion solution (4 mg collagenase I, 4.5 mg Dispase in 5 mL PBS,

Worthington, Lakewood, NJ) solution at 37 °C, 5% CO₂ for 30 min. For cells encapsulated in 3D alginate disks, each disk was incubated in isotonic 55 mM sodium citrate solution at 4 °C with gentle mixing for 10–15 min until alginate was fully dissolved. The cell solution was centrifuged for 5 min at 1200 rpm at 4 °C and the cell pellet was rinsed twice in PBS/0.5% BSA. One million cells were pelleted in 1.5 mL microcentrifuge tubes and were resuspended in 200 μ L PBS/0.5% BSA containing primary antibody and incubated for 1 h on ice in the dark. Upon centrifugation, cell pellets were rinsed twice in PBS/0.5% BSA and incubated with secondary antibody (when necessary) for 45 min on ice in the dark. Cells pellets were again rinsed twice in PBS/0.5% BSA, passed through a cell strainer to prevent cell clumps (70 μ m, BD Falcon), and transferred to FACS tubes. Primary antibodies to CD 51 (integrin subunit α_v), CD 49e (integrin subunit α_5), Cd 49b (integrin subunit α_2), and CD 29 (integrin subunit β_1) were obtained from BD Biosciences Pharmingen (San Jose, CA) and Alexa 488 anti-rat secondary was obtained from Invitrogen (Carlsbad, CA). Control experiments were performed in which cells in tissue culture were either processed with or without isotonic sodium citrate to assess effects of sodium citrate on integrin expression profile. Changes in integrin expression profile were not observed due to treatment with sodium citrate (data not shown).

FACS Analysis of Integrin Expression Profile—Samples were analyzed using the LSR II (BD Biosciences, San Jose, CA) flow cytometer and the FACSDiva software. Ten thousand events were recorded and the cell population was gated for viable cells only. Each condition was compared to control (either isotype control or cells without staining), as control experiments showed that baseline fluorescence values using either were similar. Data analysis was performed using FACSDiva and graphs were plotted using FlowJo v.6 (TreeStar Inc., Ashland, OR) software. Flow cytometry data are plotted as histograms, which normalizes the area under the curve and displays unit distribution versus FITC or PE, depending on which fluorescence molecule was used for antibody detection. The Y axis is scaled to represent the percent of cells in a given bin.

Statistical Analysis

Values represent mean and standard deviation. Statistical significance of data was assessed using one way ANOVA with respect to substrate conditions for the respective experiment (either varying RGD density or island spacing) or isotype control (for flow cytometry analysis), followed by a posthoc comparison using the Tukey test. * $p < 0.05$ between noted conditions.

Results and Discussion

Adhesion Ligand Bond Formation in 3D (FRET Studies)

The bond formation of MC3T3 preosteoblasts and clonally derived D1 stem cells in 3D alginate hydrogels was examined using a FRET technique. First, a control experiment was performed to assess whether cell–ligand interactions were specifically mediated by the RGD peptide sequence. In conditions where the MC3T3 or D1 cells were encapsulated in DS 2 alginate matrices modified with the nonsense RGE-TAMRA peptide, the degree of energy transfer was negligible (see Supporting Information, Figure S2 for D1 cells; MC3T3 data not shown), confirming specificity of the cell–ligand interaction. Previous control experiments for this assay were performed with MC3T3 preosteoblasts preincubated in soluble RGD peptides prior to encapsulation in RGD-TAMRA-modified alginate, and the degree of energy transfer was negligible as well.⁶ Inclusion of TAMRA to the RGD peptide is not expected to affect binding to integrins as the peptide sequence used in these studies, G₄RDASSK-TAMRA, was designed to minimize this possibility. The ASSK spacer is intended to allow the integrin to bind to the RGD sequence without interference from the TAMRA dye. A previous report performed TAMRA labeling of integrin receptors to investigate integrin binding to RGD sequences, and did not observe TAMRA interference with integrin-RGD binding.²³

The MC3T3 preosteoblasts and D1 stem cells were encapsulated in 3D alginate gels with increasing DS to examine the effect of increasing DS on adhesion ligand bond formation. The donor emission intensity of MC3T3s (Figure 1A) and D1 cells (Figure 1B) decreased with increasing DS. Therefore, the calculated degree of energy transfer and, correspondingly, bond formation (as determined using a standard calibration curve) increased with increasing RGD density in a similar manner for both cell types (Figure 1C [MC3T3], Figure 1D [D1 cells]). Bond formation of both MC3T3 and D1 cells increased with greater number of RGD peptides, reflecting greater cell–ligand interaction. Interestingly, the number of bonds formed by each cell type with respect to a given ligand density were similar in magnitude, and the number of bonds was not significantly influenced by the stage of differentiation of the cells.

Next, we investigated how cell-adhesion ligand bond formation was influenced by varying RGD island spacing (while maintaining the same overall number of adhesion ligands). The number of bonds formed per cell did not vary significantly with increasing RGD island spacing (36, 85, and 121 nm [overall peptide density of 6.25 mg/g alginate] or 36, 60, and 85 nm [overall peptide density of 12.5 mg/g alginate]) for MC3T3 preosteoblasts (Figure 1E) or D1 stem cells (Figure 1F). Therefore, RGD island spacing (at the ligand densities examined) did not significantly alter the number of bonds formed by MC3T3 preosteoblasts or D1 stem cells. These results indicate that the presentation of the same number of ligands, regardless of whether ligands were uniformly distributed or clustered, did not significantly effect the number of bonds formed at the cell–matrix interface over the tested range. It is possible that varying RGD presentation over an extended range of peptide densities or spacings may influence bond formation. Clearly, if the island spacing were extremely large, cell binding would be minimal. The FRET technique could be similarly employed in future experiments to probe the effects of an extended range of RGD island spacing on bond formation at the cell–matrix interface. It has been reported that membrane dynamics of integrins are altered during osteogenic differentiation of human bone marrow osteoprogenitor cells.²⁴ Integrins in undifferentiated osteoprogenitors were clustered and had slow diffusion rates, whereas osteoprogenitors undergoing osteogenic differentiation had higher diffusion rates and membrane dynamics were indistinguishable from terminally differentiated osteoblasts. The membrane-cytoskeleton interaction in stem cells and differentiated cells is different as the osteoprogenitor cytoskeleton appears to be weakly associated with membrane.²⁵ Although bond formation may be similar for preosteoblasts and stem cells at the ligand densities and RGD island spacings examined, the total bond number may not reflect the bond strength or dynamic changes in integrin binding, and these may contribute to the cell response to ECM cues. Cell–ligand interactions are a dynamic process and, hence, probing the kinetics of cell–ligand binding by using FRET to monitor bond formation over time may also be important.

FACS Analysis of Integrin Expression Profile of MC3T3 and D1 cells

Distinct integrin–ligand interactions may activate differing downstream signaling pathways,^{10,11} indicating that the identification of specific integrins expressed and utilized in adhesion ligand bond formation may be critical for understanding the cell response. The integrin profile of MC3T3 preosteoblasts, D1 stem cells and predifferentiated D1 cells were subsequently determined via flow cytometry (FACS analysis) to investigate how the integrin expression changed with differentiation. Differentiation of D1 stem cells down the osteogenic lineage was confirmed with quantification of osteocalcin, a late stage, bone specific marker (data not shown) and von Kossa staining for matrix mineralization (see Supporting Information, Figure S3). The integrin subunits examined include α_2 , α_v , α_5 , and β_1 , as these have been demonstrated to play an important role in osteoblast-ECM mediated adhesion or differentiation.^{12,26,27} The integrin expression profile of MC3T3 preosteoblasts and D1 stem cells cultured on tissue culture plastic was first examined to establish baseline integrin values. MC3T3 preosteoblasts had constitutively high levels of α_v , α_5 , and β_1 expression, but negligible α_2 levels (Figure 2A,

B [data not shown for α_2 and β_1]). D1 stem cells expressed high levels of α_v and β_1 , but negligible α_2 and α_5 (Figure 2C,D, respectively; values shown in Table 1). However, as D1 stem cells were differentiated toward osteoblasts, α_v and α_5 expression was upregulated 2-fold and 7-fold, respectively (Figure 2C,D, shaded area under curve), and these integrin receptor levels closely approximated those observed for MC3T3 preosteoblasts (Table 1). These results are in agreement with studies showing that α_v and α_5 integrin subunits were upregulated in human osteoblasts as compared to human mesenchymal stem cells²⁸ and that BMP-2 induction of human osteoblasts also increased α_v expression and osteoblast adhesion to osteopontin and vitronectin.²⁹ The α_v integrin subunit mainly mediates adhesion, whereas the α_5 integrin subunit is more highly expressed in differentiated cells,^{28,30,31} and this may in part explain why levels of α_5 were higher in MC3T3 preosteoblasts and differentiated D1 cells as compared to uncommitted D1 stem cells. However, integrin overexpression may inhibit differentiation as $\alpha_v\beta_3$ overexpression has been shown to inhibit mineralization of MC3T3 preosteoblasts.²⁶ Upregulation of α_v and α_5 in differentiated cells, perhaps in concert with other integrin receptors, may contribute to the greater response of committed cell populations to ECM cues.

3

To determine which integrins were engaged in gels presenting RGD ligand, the integrin expression profiles of MC3T3 preosteoblasts and D1 stem cells cultured on top of RGD presenting alginate (DS 2) substrates were examined (2D culture). For MC3T3 preosteoblasts, the ratio of α_v to α_5 expression was significantly upregulated, compared to culture on tissue culture plastic, and reflects a switch to α_v as the primary receptor binding to RGD-modified substrates ([DS 2], Figure 3A,B; values shown in Table 2). The α_v and α_5 integrin expression of the preosteoblasts did not vary with DS of the substrate, as the levels remained similar when cultured on alginate with DS 8 (see Supporting Information, Table S1). Although the expression of the α_5 integrin subunit was downregulated when cells were cultured on RGD presenting substrates, α_5 levels (although low) were higher in the MC3T3 preosteoblasts (Figure 3B) as compared to the D1 stem cells, where α_5 expression was negligible (Figure 3D). The α_v and α_5 integrin expression level of the D1 stem cells were similar on RGD substrates and tissue culture plastic (Figure 3C,D, respectively).

The integrin expression profile of MC3T3 preosteoblasts cultured on RGD presenting substrates was remarkably different from those cultured on tissue culture plastic. As the α_5 expression of preosteoblasts was dramatically downregulated, the α_v integrin subunit would presumably act as the primary receptor binding to RGD-modified substrates. Although both fibronectin ($\alpha_5\beta_1$) and vitronectin ($\alpha_v\beta_3$) receptors recognize the RGD sequence,^{32,33} the $\alpha_v\beta_3$ integrin binds better to linear RGD peptides than $\alpha_5\beta_1$ integrin and may explain the switch from α_v and α_5 expression to predominantly α_v integrin subunit on RGD presenting substrates. The expression of α_v integrin subunits for D1 stem cells were approximately half the levels observed for MC3T3 preosteoblasts and α_5 expression was negligible. Furthermore, α_v and α_5 expression of D1 stem cells was not altered whether cultured on RGD presenting substrates or tissue culture plastic. Regardless of the stage of cell differentiation, the α_v was the primary receptor expressed and presumably mediating adhesion to the surface of RGD-modified alginate. It would be interesting to examine integrin expression levels at longer time points (e.g., 1 or 2 weeks when cells begin synthesizing natural ECM) and examine the relative α_v and α_5 levels in preosteoblasts as compared to stem cells. It is possible that during longer term culture, as the cells differentiate and synthesize and assemble their own ECM, the cells re-express or upregulate α_5 integrins. Investigation of a broader integrin expression profile of committed versus progenitor cells would also be useful for examining how the stage of differentiation of the cell influences integrin expression, cell-matrix interactions, and subsequent cell response.

For studies aiming to promote the differentiation of stem cells for tissue regeneration, examining the specific integrin subunits involved in integrin–adhesion ligand interactions in 3D culture are particularly relevant. Flow cytometry analysis of integrin expression in 3D culture was performed by encapsulating cells in RGD presenting alginate matrices at a density of 5 million cells/mL alginate (to minimize cell–cell contact such that cell–matrix interactions dominate). Flow cytometry analysis of MC3T3 preosteoblasts encapsulated in DS 2 RGD-modified alginate disks indicate that there are high levels of α_v and α_5 expression, two integrin subunits important in osteoblast differentiation. Whereas negligible α_v and α_5 expression were observed in 2D culture on RGD-modified alginate substrates, an upregulation of these integrin subunits was observed when cells were cultured in 3D using the same alginate substrate (DS 2 RGD) at the same time point (day 3; Figure 4A,B). Similarly, flow cytometry analysis of D1 stem cells encapsulated in DS 2 RGD alginate matrices and cultured for 3 days also demonstrated high expression of α_v and α_5 integrin expression (Figure 4C,D). The integrin expression of both cell types (Table 3) did not vary at the time points examined (16 h and 3 days). Whereas α_v and α_5 integrin subunit levels were low for both cell types when cultured on RGD presenting substrates, these integrin subunits were dramatically upregulated in 3D culture regardless of the stage of differentiation. These results may reflect differential integrin activation due to the transition from a 2D to 3D cellular environment. It is widely known that cells respond differently in 2D versus 3D culture, and in fact, 3D cell culture typically better reflects the *in vivo* cell microenvironment and may be a more relevant system to study complex cell behavior.^{34–36} In these studies, 3D cell culture better recapitulates the native microenvironment and promotes cell differentiation, as shown by the upregulation of the α_5 integrin subunit. The α_5 integrin subunit may be critical for differentiation and has been demonstrated to be important in mediating 3D matrix interactions.³⁷ Mimicking the natural 3D cell microenvironment may enhance the secretion and binding of different cytokines (via more efficient trapping and localization of cell-secreted ECM), which can induce integrin expression and modulate matrix binding. Overall, the results here altogether indicate that the cell integrin expression profile is dependent on the stage of differentiation (on tissue culture plastic), the substrate properties (tissue culture vs RGD presenting substrate), and whether the cell was cultured on a 2D substrate or encapsulated within a 3D matrix.

Although no differences were observed between the integrin expression profile of preosteoblasts and the stem cells encapsulated in the 3D substrates examined, it would be interesting to further investigate the integrin expression profile over time (e.g., several hours to several weeks post encapsulation) to determine whether integrin expression continues to change as cells accumulate a new extracellular matrix.

In this study, we have examined bond formation at the cell–matrix interface, and the integrins involved in these bonds, to better understand why progenitor versus committed cell types respond differently to matrix cues. Expression levels of α_v and α_5 integrin subunits, important in mediating RGD adhesion and differentiation, were found to vary with differentiation stage on tissue culture and adhesion ligand presentation in 2D. However, preosteoblasts and stem cells encapsulated within 3D RGD presenting matrices were observed to form similar bond numbers at the ligand densities and RGD island spacings examined. In addition, α_v and α_5 integrin subunit levels were similar in magnitude regardless of the stage of differentiation in 3D RGD presenting matrices. It was previously reported that adhesion ligand presentation (e.g., density, distribution) regulated the proliferation of MC3T3 preosteoblasts, whereas the proliferation of D1 stem cells was less sensitive to these cues.³ Although integrin–adhesion ligand bond formation were observed to be similar for preosteoblasts and stem cells in 3D culture, it is possible that integrin–adhesion ligand bond formation may be altered at different time points. Furthermore, integrin–adhesion ligand bond formation may not reflect variations in bond stability or strength. There is a weaker association between the membrane and cytoskeleton in stem cells^{38,39} and differences in membrane mechanics between progenitor

and committed cell types may contribute to the differential cell response to matrix cues. Variation in cell shape, integrin clustering, and subsequent downstream signaling events may also influence the cell response.

Conclusions

FRET is a powerful technique to quantify cell–ligand interaction and to examine how the bond formation is regulated by adhesion ligand presentation (e.g., RGD ligand density and spacing). Both preosteoblast and stem cell bond formation increased with greater adhesion ligand density, but was not significantly altered due to changes in RGD island spacing. In parallel, flow cytometry analysis of MC3T3 preosteoblasts and D1 stem cells showed that the integrin expression profile of the two cell types differed, likely due to the differences in the stage of differentiation of these cells, and 3D culture dramatically altered integrin expression as compared to 2D culture. This combined approach using a FRET technique to quantify cell–ligand bond formation, as well as flow cytometry to elucidate specific integrin–ligand interactions, contributes significantly to our understanding of cell–ECM interactions in this system and how adhesion is governed by the stage of cell differentiation. These studies will inform biomaterial design strategies for stem cell transplantation and cell-based therapies with these and similar cells, and these techniques will likely be of broad utility with other cell types and materials systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH (R37 DE13033), the U.S. Army Research Laboratory, and the U.S. Army Research Office Grant DAAD190310168.

References and Notes

1. Oyajobi BO, Lomri A, Hott M, Marie PJ. Isolation and characterization of human clonogenic osteoblast progenitors immunoselected from fetal bone marrow stroma using STRO-1 monoclonal antibody. *J Bone Miner Res* 1999;14:351–61. [PubMed: 10027900]
2. Fromigue O, Marie PJ, Lomri A. Differential effects of transforming growth factor β 2, dexamethasone, and 1,25-dihydroxyvitamin D on human bone marrow stromal cells. *Cytokine* 1997;9:613–23. [PubMed: 9245490]
3. Hsiong SX, Carampin P, Kong HJ, Lee KY, Mooney DJ. Differentiation stage alters matrix control of stem cells. *J Biomed Mater Res A* 2007;4:1501–6.
4. Alsberg E, Anderson KW, Albeiruti A, Franceschi RT, Mooney DJ. Cell-interactive alginate hydrogels for bone tissue engineering. *J Dent Res* 2001;80:2025–9. [PubMed: 11759015]
5. Simmons CA, Alsberg E, Hsiong S, Kim WJ, Mooney DJ. Dual growth factor delivery and controlled scaffold degradation enhance in vivo bone formation by transplanted bone marrow stromal cells. *Bone* 2004;35:562–9. [PubMed: 15268909]
6. Kong HJ, Boonthekul T, Mooney DJ. Quantifying the relation between adhesion ligand-receptor bond formation and cell phenotype. *Proc Natl Acad Sci US A* 2006;103:18534–9.
7. Berney C, Danuser G. FRET or no FRET: A quantitative comparison. *Biophys J* 2003;84:3992–4010. [PubMed: 12770904]
8. Kubitscheck U, Kircheis M, Schweitzer-Stenner R, Dreybrodt W, Jovin TM, Pecht I. Fluorescence resonance energy transfer on single living cells. Application to binding of monovalent haptens to cell-bound immunoglobulin E. *Biophys J* 1991;60:307–18. [PubMed: 1832974]

9. Ha T, Enderle T, Ogletree DF, Chemla DS, Selvin PR, Weiss S. Probing the interaction between two single molecules: Fluorescence resonance energy transfer between a single donor and a single acceptor. *Proc Natl Acad Sci US A* 1996;93:6264–8.
10. Kundu AK, Putnam AJ. Vitronectin and collagen I differentially regulate osteogenesis in mesenchymal stem cells. *Biochem Biophys Res Commun* 2006;347:347–57. [PubMed: 16815299]
11. Gilbert M, Giachelli CM, Stayton PS. Biomimetic peptides that engage specific integrin-dependent signaling pathways and bind to calcium phosphate surfaces. *J Biomed Mater Res A* 2003;67:69–77. [PubMed: 14517863]
12. Takeuchi Y, Suzawa M, Kikuchi T, Nishida E, Fujita T, Matsumoto T. Differentiation and transforming growth factor-beta receptor down-regulation by collagen- $\alpha 2\beta 1$ integrin interaction is mediated by focal adhesion kinase and its downstream signals in murine osteoblastic cells. *J Biol Chem* 1997;272:29309–16. [PubMed: 9361011]
13. Xiao G, Gopalakrishnan R, Jiang D, Reith E, Benson MD, Franceschi RT. Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. *J Bone Miner Res* 2002;17:101–10. [PubMed: 11771655]
14. Rowley JA, Madlambayan G, Mooney DJ. Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials* 1999;20:45–53. [PubMed: 9916770]
15. Lee KY, Alsberg E, Hsiong SX, Comisar WA, Linderman JJ, Ziff R, Mooney DJ. Nanoscale adhesion ligand organization regulates osteoblast proliferation and differentiation. *Nano Lett* 2004;4:1501–6.
16. Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J Cell Biol* 1983;96:191–8. [PubMed: 6826647]
17. Nakatani Y, Tsunoi M, Hakeda Y, Kurihara N, Fujita K, Kumegawa M. Effects of parathyroid hormone on cAMP production and alkaline phosphatase activity in osteoblastic clone MC3T3-E1 cells. *Biochem Biophys Res Commun* 1984;123:894–8. [PubMed: 6091653]
18. Franceschi RT, Iyer BS. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *J Bone Miner Res* 1992;7:235–46. [PubMed: 1373931]
19. Li X, Cui Q, Kao C, Wang GJ, Balian G. Lovastatin inhibits adipogenic and stimulates osteogenic differentiation by suppressing PPAR 2 and increasing Cbfa1/Runx2 expression in bone marrow mesenchymal cell cultures. *Bone* 2003;33:652–9. [PubMed: 14555271]
20. Li X, Jin L, Cui Q, Wang GJ, Balian G. Steroid effects on osteogenesis through mesenchymal cell gene expression. *Osteoporosis Int* 2005;16:101–8.
21. Devine MJ, Mierisch CM, Jang E, Anderson PC, Balian G. Transplanted bone marrow cells localize to fracture callus in a mouse model. *J Orthop Res* 2002;20:1232–9. [PubMed: 12472234]
22. Diduch DR, Coe MR, Joyner C, Owen ME, Balian G. Two cell lines from bone marrow that differ in terms of collagen synthesis, osteogenic characteristics, and matrix mineralization. *J Bone Joint Surg Am* 1993;75:92–105. [PubMed: 8419395]
23. Goennenwein S, Tanaka M, Hu B, Moroder L, Sackmann E. Functional incorporation of integrins into solid supported membranes on ultrathin films of cellulose: impact on adhesion. *Biophys J* 2003;85:646–55. [PubMed: 12829518]
24. Chen H, Titushkin I, Strosio M, Cho M. Altered membrane dynamics of quantum dot-conjugated integrins during osteogenic differentiation of human bone marrow derived progenitor cells. *Biophys J* 2007;92:1399–408. [PubMed: 17114225]
25. Titushkin I, Cho M. Distinct membrane mechanical properties of human mesenchymal stem cells determined using laser optical tweezers. *Biophys J* 2006;90:2582–91. [PubMed: 16399828]
26. Cheng SL, Lai CF, Blystone SD, Avioli LV. Bone mineralization and osteoblast differentiation are negatively modulated by integrin $\alpha(v)\beta 3$. *J Bone Miner Res* 2001;16:277–88. [PubMed: 11204428]
27. Moursi AM, Globus RK, Damsky CH. Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation in vitro. *J Cell Sci* 1997;110(18):2187–96. [PubMed: 9378768]
28. Bennett JH, Carter DH, Alavi AL, Beresford JN, Walsh S. Patterns of integrin expression in a human mandibular explant model of osteoblast differentiation. *Arch Oral Biol* 2001;46:229–38. [PubMed: 11165569]

29. Lai CF, Cheng SL. $\alpha v \beta$ integrins play an essential role in BMP-2 induction of osteoblast differentiation. *J Bone Miner Res* 2005;20:330–40. [PubMed: 15647827]
30. Pistone M, Sanguineti C, Federici A, Sanguineti F, Defilippi P, Santolini F, Querze G, Marchisio PC, Manduca P. Integrin synthesis and utilization in cultured human osteoblasts. *Cell Biol Int* 1996;20:471–9. [PubMed: 8931314]
31. Petrie TA, Capadona JR, Reyes CD, Garcia AJ. Integrin specificity and enhanced cellular activities associated with surfaces presenting a recombinant fibronectin fragment compared to RGD supports. *Biomaterials* 2006;27:5459–70. [PubMed: 16846640]
32. Pytela R, Pierschbacher MD, Ruoslahti E. A 125/115-kDa cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic acid adhesion sequence derived from fibronectin. *Proc Natl Acad Sci US A* 1985;82:5766–70.
33. Ruoslahti E. RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol* 1996;12:697–715. [PubMed: 8970741]
34. Yamada KM, Cukierman E. Modeling tissue morphogenesis and cancer in 3D. *Cell* 2007;130:601–10. [PubMed: 17719539]
35. Pampaloni F, Reynaud EG, Stelzer EH. The third dimension bridges the gap between cell culture and live tissue. *Nat Rev Mol Cell Biol* 2007;8:839–45. [PubMed: 17684528]
36. Smalley KS, Lioni M, Herlyn M. Life isn't flat: Taking cancer biology to the next dimension. *In Vitro Cell Dev Biol : Anim* 2006;42:242–7. [PubMed: 17163781]
37. Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. *Science* 2001;294:1708–12. [PubMed: 11721053]
38. Sun S, Titushkin I, Cho M. Regulation of mesenchymal stem cell adhesion and orientation in 3D collagen scaffold by electrical stimulus. *Bioelectrochemistry* 2006;69:133–41. [PubMed: 16473050]
39. Dai J, Sheetz MP. Membrane tether formation from blebbing cells. *Biophys J* 1999;77:3363–70. [PubMed: 10585959]

Figure 1.

FRET to measure degree of energy transfer between fluorophores on preosteoblasts and stem cells and those in RGD ligands presented from 3D alginate substrates in which cells are encapsulated. Donor emission intensity of MC3T3 preosteoblasts (A) and D1 stem cells (B) in 3D RGD-TAMRA-modified alginate gels (degree of substitution [DS] 2, island spacing 36 nm) [donor control (open circles), FRET condition (black squares)]. Bond formation of MC3T3 preosteoblasts (C) and D1 stem cells (D) as DS is increased in 3D RGD-TAMRA-modified alginate gels. Bond formation of MC3T3 (E) and D1 (F) in 3D RGD-modified alginate gels as RGD island spacing is varied from 36–121 nm for RGD density of 1 mol (square) and 2 moles RGD-TAMRA per mole alginate (circle). For 1 mol RGD/mol alginate, spacing is varied by mixing DS 10 and DS 20 RGD alginate in a 1:9 and 1:19 ratio with unmodified alginate to create RGD island spacing of 85 and 121 nm, respectively. For 2 moles RGD/mol alginate, spacing is varied by mixing DS 10 and DS 20 RGD alginate in a 1:4 and 1:9 ratio with unmodified alginate to create RGD island spacing of 60 and 85 nm, respectively. For RGD island spacing of 36 nm, RGD alginate was used without dilution with unmodified alginate (DS 0). Values represent mean and standard deviation ($n = 4$ or 5). $*p < 0.05$; values represent mean and standard deviation ($n = 4$ or 5).

Figure 2.

α_v and α_5 integrin subunit expression of MC3T3 preosteoblasts (A, B) and D1 stem cells (C, D), cultured on tissue culture plastic. (A, B) Flow cytometry was used to determine the relative integrin expression of antibody treated MC3T3 cells (black) compared to isotype control (grey). (C, D) α_v and α_5 integrin expression of predifferentiated D1 cells (shaded area, D1 stem cells differentiated toward osteoblasts), compared to undifferentiated D1 cells (black) and isotype control (grey).

Figure 3. α_v and α_5 integrin subunit expression of MC3T3 preosteoblasts (A, B) and D1 stem cells (C, D), cultured on RGD presenting alginate substrate (shaded) [(DS 2, 12.5 mg RGD/g alginate)] for three days, or on tissue culture plastic (black). Isotype control (grey) is also shown.

Figure 4. α_V and α_5 integrin subunit expression of MC3T3 preosteoblasts (A, B) and D1 stem cells (C, D), encapsulated in (shaded area) or cultured on top of RGD presenting alginate substrate (black line; DS 2, 12.5 mg RGD/g alginate) for three days. Flow cytometry was used to determine the relative integrin expression compared to isotype control (grey).

Table 1

Overall Integrin Expression, as Percentage of Viable Cell Population, of Subunits α_2 , α_v , α_5 , and β_1 for MC3T3, D1, and Predifferentiated D1 Cells [D1 (A/B)] Cultured on Tissue Culture Plastic^a

integrin subunit		MC3T3, % of viable cell population	D1, % of viable cell population	D1 (A/B), % of viable cell population
α_2	CD 49b	2	3	2
α_v	CD 51	85	36	68
α_5	CD 49e	74	8	59
β_1	CD 29	93	98	99

^aFACS data gated for viable cells only.

Table 2

Overall Integrin Expression, as Percentage of Viable Cell Population, of α_2 , α_v , α_5 , and β_1 for MC3T3, D1, and Predifferentiated D1 Cells [D1 (A/B)] Cultured on RGD-Modified Alginate Substrates (DS 2, Day 3)^a

integrin subunit		MC3T3, % of viable cell population	D1, % of viable cell population	D1 (A/B), % of viable cell population
α_2	CD 49b		4	7
α_v	CD 51	89	37	46
α_5	CD 49e	11	1	9
β_1	CD 29		89	81

^aFACS data gated for viable cells only. α_2 and β_1 integrin expression of MC3T3 were not examined.

Table 3

Overall Integrin Expression, as Percentage of Viable Cell Population, of Subunits α_2 , α_v , α_5 , and β_1 for MC3T3 and D1 Cells Encapsulated in RGD-Modified Alginate Substrates (DS 2, day 3)^a

3D matrix	integrin subunit	MC3T3, % of viable cell population	D1, % of viable cell population
DS 2 RGD	α_v , CD 51	89	71
	α_5 , CD 49e	95	96

^aFACS data gated for viable cells only.