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Cell-matrix interactions and dynamic mechanical loading influence chondrocyte gene expression and bioactivity in PEG-RGD hydrogels

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Abstract

The pericellular matrix (PCM) surrounding chondrocytes is thought to play an important role in transmitting biochemical and biomechanical signals to the cells, which regulates many cellular functions including tissue homeostasis. To better understand chondrocytes interactions with their PCM, 3D poly(ethylene glycol) (PEG) hydrogels containing Arg-Gly-Asp (RGD), the cell adhesion sequence found in fibronectin and which is present in the PCM of cartilage, were employed. RGD was incorporated into PEG hydrogels via tethers at 0.1, 0.4, and 0.8 mM concentrations. Bovine chondrocytes were encapsulated in the hydrogels, subjected to dynamic compressive strains (0.3Hz, 18% amplitude strain) for 48h, and their response assessed by cell morphology, ECM gene expression, cell proliferation, and matrix synthesis. Incorporation of RGD did not influence cell morphology under free swelling conditions. However, the level of cell deformation upon an applied strain was greater in the presence of RGD. In the absence of dynamic loading, RGD appears to have a negative effect on chondrocyte phenotype as seen by a 4.7-fold decrease in collagen II/collagen I expressions in 0.8 mM RGD constructs. However, RGD had little effect on early responses of chondrocytes (i.e., cell proliferation and matrix synthesis/deposition). When isolating RGD as a biomechanical cue, cellular response was very different. Chondrocyte phenotype (collagen II/collagen I ratio) and proteoglycan synthesis were enhanced with higher concentrations of RGD. Overall, our findings demonstrate that RGD ligands enhance cartilage-specific gene expression and matrix synthesis, but only when mechanically stimulated, suggesting that cell-matrix interactions mediate chondrocyte response to mechanical stimulation.

Keywords

chondrocytes; RGD; dynamic loading; hydrogel; cell-matrix interactions

Introduction

The extracellular matrix of cartilage is comprised primarily of type II collagen and aggrecan. However, chondrocytes, the cells responsible for maintaining cartilage homeostasis, are surrounded by a very different matrix referred to as the pericellular matrix (PCM). The PCM

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is rich in a plethora of different matrix molecules including collagen types II, VI and IX, fibronectin, aggrecan, hyaluronan and decorin. The PCM is thought to play an important role in transmitting both biochemical and biomechanical cues from the extracellular matrix to the cell through “outside-in” signaling mechanisms [1]. However the exact role of the PCM is not well-understood.

It is well-known that cells interact with their surrounding matrix through cell surface receptors and/or integrins located on the cell membrane [2]. The oligopeptide sequence, Arg-Gly-Asp (RGD) is one of the most widely studied cell adhesion moieties for its presence in a number of proteins, most notably fibronectin [3]. Several studies have shown that chondrocyte attachment to fibronectin can be blocked by the addition of soluble RGD indicating that chondrocyte adhesion to fibronectin is mediated via RGD [4,5]. Furthermore, Alsberg *et al.* [6] demonstrated that incorporating RGD into alginate hydrogels enhanced cartilaginous tissue formation *in vivo* suggesting a potential role for RGD in cartilage regeneration.

One mechanism by which cells sense mechanical forces is through their interaction with ligands in the surrounding matrix, which result in integrin-mediated mechanotransduction events. Several studies have explored the role of RGD in chondrocyte mechanotransduction [7–11]. For example, when chondrocytes were cultured on the surface of RGD-modified alginate hydrogels, chondrocyte morphology and organization of actin stress fibers within the cell was highly dependent on the substrate stiffness suggesting that RGD provides a mechanism by which chondrocytes are able to sense their underlying substrate [12]. Wright *et al.* [13] demonstrated that mechanical stimulation of chondrocytes in 2D cultures can lead to rapid activation of intracellular signaling cascades involving regulation of ion channels and membrane hyperpolarization, pathways which can lead to the secretion of growth factors and cytokines [14]. This response has been shown to be initiated by integrin-mediated mechanotransduction events involving chondrocyte attachment to fibronectin via $\alpha 5 \beta 1$ integrins. To elucidate the mechanotransduction pathways in chondrocytes in an environment that better mimics that of cartilage, a number of groups have encapsulated chondrocytes in 3D agarose hydrogels [10,11,15–20]. For example, when cell-laden agarose gels were subjected to physiological dynamic compressive strains, loading enhanced cell proliferation and proteoglycan synthesis. This response was blocked by soluble RGD indicating that the enhanced cellular response was due to cellular interactions with RGD containing proteins and/or proteins that bind to $\alpha 5 \beta 1$ integrins [10,11]. Together, these studies demonstrate that RGD can play an important role in mediating cellular response through integrin-mediated mechanotransduction events in chondrocytes.

In tissue engineering strategies, RGD has been widely employed as a cell-adhesion moiety to promote cell attachment and adhesion to cell-based scaffolds [10–12,18,20,21–25]. In particular, poly(ethylene glycol) (PEG)-based hydrogels have been employed as 3D cell carriers for a wide range of tissue engineering applications [21,22,24–28]. Because PEG is bioinert and does not interact with proteins or cells, strategies have been developed where RGD is tethered to the network to promote cell attachment and adhesion to the PEG hydrogel [25]. A number of studies have demonstrated that the incorporation of RGD tethers significantly influences cellular functions for a variety of cell types including osteoblasts [21,22,29], mesenchymal stem cells [26,30], endothelial cells [27,31], and smooth muscle cells [28]. For example, osteoblast attachment, spreading and mineralization increased in a dose-dependent manner when RGD was incorporated into PEG hydrogels [21]. For attachment dependent cells, the incorporation of tethered RGD into PEG hydrogels was shown to be vital in maintaining the survival of human mesenchymal stem cell within the hydrogels [26]. Together, these studies suggest that RGD presented in this tethered manner may help to mimic the native tissue environment.

Because PEG does not promote cell interactions, it provides an excellent base which to incorporate matrix analogs into the network in a highly controlled manner as a way to explore the role of matrix components in mediating cellular functions. A number of studies, as described above, have shown enhanced cellular functions when RGD is incorporated into PEG hydrogels. The impact of mechanical forces, however, has not been studied in these PEG hydrogels systems containing matrix analogs. Therefore, our goal for the present study was to employ 3D PEG hydrogels as a platform to investigate the role of cell-matrix interactions involved in chondrocyte mechanotransduction by systematically incorporating RGD into the PEG hydrogel network and subjecting the hydrogels to dynamic compressive loading. By covalently attaching RGD to the PEG network, we can begin to develop a system that better mimics the native PCM microenvironment and in particular, chondrocyte interactions with fibronectin. In addition, findings from this study will provide insights into designing 3D hydrogel matrices for cartilage tissue engineering applications where mechanical stimulation is employed.

Specifically, bovine chondrocytes were encapsulated in PEG gels where the crosslinking density was fixed but the RGD concentration was varied (0–0.8 mM). The cell-laden hydrogels were subjected to dynamic compressive strains for 48 hours to assess the role of RGD in chondrocyte mechanotransduction events. We chose short culture times to minimize the contribution of any elaborated PCM deposited by the encapsulated chondrocytes. The dynamically strained hydrogel constructs were compared to control constructs, which were not subjected to dynamic loading but which were cultured in the bioreactor under similar conditions. Chondrocyte morphology was assessed in unstrained hydrogels to determine if the presence of RGD influenced cell shape or under an applied strain to determine if the presence of RGD influenced the level of cellular deformation experienced by the cells during dynamic loading. Chondrocyte response was assessed as a function of RGD concentration in the absence of dynamic compressive strains to investigate the role of RGD in cell-matrix interactions and in the presence of dynamic strains to investigate the involvement of RGD in integrin-mediated mechanotransduction. Cellular responses were measured by extracellular matrix gene expression, cell proliferation, and matrix deposition for proteoglycans and collagen. Gene expression for collagen type I, collagen type II, and aggrecan was assessed using real time quantitative PCR where the ratio of collagen type II/collagen type I expression was employed as an indicator of chondrocyte differentiation. Cell proliferation was measured by [³H]-thymidine incorporation into newly synthesized DNA. Matrix deposition was determined by [³⁵SO₄²⁻] incorporation into newly synthesized proteoglycans and hydroxyproline content as a measure of collagen deposition.

2. Methods

2.1 Hydrogel Preparation

Poly(ethylene glycol) (3000 MW, Fluka, USA) was dissolved in methylene chloride and reacted with excess chilled acryloyl chloride and triethylamine under argon for 24 h at 4°C. The final product, poly(ethylene glycol) diacrylate (PEGDA) was precipitated twice in ethyl ether and vacuum filtered to remove any impurities. The area under the curve for the vinyl resonances (δ = 5.8 ppm, δ =6.2, and δ =6.4 ppm) associated with the acrylate substitution was compared to that of the methylene protons (δ =4.3 ppm) associated with the PEG backbone. The degree of acrylate substitution was 88% as determined by ¹H NMR (Varian VYR-500, California, USA).

NH₂-Tyr-Arg-Gly-Asp-Ser-COOH (YRGDS) (Bachem, California, USA) (1 mg/ml) in a 10% molar excess was reacted with acryloyl-PEG-*N*-hydroxysuccinimide (ACR-PEG-NHS) (MW 3,400, Nektar Therapeutics, Alabama, USA) (10 mg/ml) in a 50 mM bicarbonate buffer (pH=8.4) at room temperature for 2 hours. The extent of reaction was determined by assaying

for unreacted RGD through the presence of primary amines as detected by fluoraldehyde reagent (Peirce, Illinois, USA); 80% of acryloyl-PEG-NHS was conjugated with RGD. The final product, acryloyl-PEG-RGD (ACR-PEG-RGD) was dialyzed overnight to remove the unreacted RGD and recovered by lyophilization for 24 hours.

2.2 Chondrocyte Isolation

Full depth articular cartilage was removed under sterile conditions from metacarpalphalangeal joints of a total of six 1–2 year adult steers within several hours after slaughter (Arapahoe Foods, Colorado, USA). The cartilage slices were washed in phosphate buffered saline (PBS-1X, Invitrogen, California, USA) supplemented with 1% penicillin-streptomycin (PBS-P/S), diced finely and enzymatically digested by 0.2% collagenase type II (Worthington Biochemical Corp, New Jersey, USA) in Dulbecco's Minimal Essential Medium (DMEM, Invitrogen, California, USA) containing 5% fetal bovine serum (Invitrogen, California, USA) for a maximum of 16 hours at 37°C/5% CO₂ to solubilize the extracellular and pericellular matrices. The isolated chondrocytes were centrifuged three times at 1200 rpm for 10 minutes and resuspended in PBS-P/S. Cell viability was determined by trypan-blue exclusion and was greater than 94% prior to encapsulation.

2.3 Chondrocyte Encapsulation

PEGDA macromer (10% (w/w)) and 0.05% (w/w) photoinitiator (1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one, Ciba Specialty Chemical, Georgia, USA) were dissolved in PBS (pH 7.4). RGD macromer (ACR-PEG-RGD) was then added to the PEGDA macromer solution at concentrations of 0.1, 0.4, or 0.8 mM RGD. To create the cell-laden hydrogels, isolated chondrocytes were suspended in the macromer/initiator solution at a concentration of 50×10^6 cells/mL and exposed to 365 nm light at ~ 2 mW/cm² for 10 min to entrap the cells within cylindrical PEG hydrogel constructs (5mm in diameter and 5mm in height). Cell seeded PEG hydrogels (PEG-only) and PEG hydrogels containing different RGD concentrations (0.1, 0.4, 0.8mM) were cultured under free swelling conditions for 24 hours within standard 24-well tissue culture plates containing 1.5 mL/well of chondrocyte media (DMEM supplemented with 10mM HEPES, 0.1M non-Essential Amino Acids, 0.4 mM L-proline, 50mg/L of L-ascorbic acid, 1X P/S 10% FBS, and 0.5µg/mL of fungizone; Invitrogen, California, USA). A scrambled peptide (YRDGS) was also tested in which no differences were observed in chondrocyte response when compared to the PEG-only gels in the presence or absence of load (data not shown).

2.4 Hydrogel Properties

Cylindrical PEG-only and PEG-RGD hydrogels (5mm in height and diameter) without cells were swollen to equilibrium in PBS at 37°C and 5% CO₂ for 24 hours. The swollen mass of the gel was measured and then lyophilized for 24 hours to determine the dry polymer mass. The equilibrium volumetric swelling ratio (Q) was determined from the equilibrium mass swelling ratio as described elsewhere [32]. The tangent compressive modulus was determined using a mechanical tester (Bose LM1 Test Bench, Minnesota, USA) in unconfined compression equipped with non-porous platens. A constant strain rate of 0.45 mm/s, similar to the frequency applied during dynamic loading of the hydrogel constructs, was applied to the gels and the resulting stress recorded. A sample size of 5 was used.

2.5 Mechanical Loading

The experimental approach is shown in Figure 1a. Hydrogel constructs were placed individually into wells of a standard 24-well plate with 1.5 mL chondrocyte media/well and cultured under free swelling conditions for 24 hours. After the initial 24 hour culture period, the constructs were placed into a custom mechanical loading apparatus [33]. Culture medium

in each well was replaced with chondrocyte medium supplemented with 1 $\mu\text{Ci/mL}$ of [^3H]-thymidine and 10 $\mu\text{Ci/mL}$ of [$^{35}\text{SO}_4^{2-}$] (Perkin Elmer, CT, USA). Each custom bioreactor is equipped with 24 impermeable pins associated with each well and construct. The hydrogel constructs were subjected to unconfined compressive strains with an initial tare strain of 3%, associated with the platens. Dynamic compressive strains were applied at a frequency of 0.3 Hz in a sinusoidal waveform from 3 to 18% for 48 hours. Control samples were also placed in the bioreactor, but were not subjected to dynamic loading.

2.6 Mechanical Properties of Cell-Laden Constructs

The tangent compressive modulus for the cell-laden constructs was assessed prior to being placed into the bioreactor, i.e., after the initial 24 hour free swelling period, and immediately after the 48 hour period in the bioreactor for the control or loaded constructs. The modulus was measured as described above in *section 2.4* for the acellular hydrogels. A sample size of 3 was used.

2.7 Chondrocyte Deformation

Chondrocytes were visualized using the fluorescent membrane integrity assay, LIVE/DEAD® Assay (Invitrogen, California, USA) where live cells fluoresce green and dead cells fluoresce red. Specifically, hydrogel constructs were treated with 2 μM calcein-AM and 2 μM ethidium homodimer and incubated at 5% CO_2 and 37°C for 20–30 minutes. Chondrocyte viability was assessed through the course of the experiment and found to be ~70% (unpublished results). There were no statistical differences in chondrocyte viability among all experimental conditions. Chondrocyte deformation was assessed after the initial 24 hour free swelling period prior to being placed into the bioreactor and after the 48 hour period within the bioreactor for both the control and loaded constructs. The gels were placed in a custom-built cell straining device similar to that described by Knight *et al.* [34], which sits on the stage of an inverted laser scanning confocal microscope (Zeiss LSM 510 confocal microscope). Constructs were subjected to either 0% strain or 18% compressive strain. Images of chondrocytes within the hydrogel were acquired at full width half maximum height with a 40 $\times$ oil immersion objective. A total of three images at different regions within the hydrogel were acquired and 3 gels were analyzed per condition. Approximately 18 cells were analyzed for each experimental condition. Before and after removal from the bioreactor, the control and loaded specimen were removed and cell morphology was quantified by a cell diameter ratio (x/y) [34]. A separate set of control and loaded specimen were removed from the bioreactor and cultured under free swelling conditions for an additional six hours prior to measuring cell diameter ratios. For constructs cultured under free swelling conditions for 24 hours, cell diameter ratio was defined by the ratio of the minimum cell diameter (x) to the maximum cell diameter (y). For constructs which were cultured in the bioreactor for 48 hours or allowed to free swell post-loading for 6 hours, cell diameter ratio was defined by the ratio of cell diameter parallel to the applied strain (x) to the cell diameter perpendicular to the applied strain (y).

2.8 Gene Expression using Real-Time PCR

After 48 hours in the bioreactor, control and dynamically loaded constructs were removed and total RNA isolated [35]. Total RNA content was quantified using Quant-iT RiboGreen Kit (Invitrogen, California, USA) and diluted with 1 $\times$ Tris EDTA buffer (pH=7.5) to a final concentration of 10 ng/ μL . A known amount (50ng) of total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Biorad, California, USA) and a thermal cycler (Eppendorf Mastercycler Personal). Real-time PCR was performed (Applied Biosystems 7500 FAST Real Time PCR) using custom designed primers and the iQ™ SYBR® Green Supermix (Biorad, California, USA). The following primers were used: collagen II sense (5'-CCC GAA CCC AGA ACC AAC AC-3') and antisense (5'-ATG GGC GTC AGG TCA GGT C-3'); collagen

1 sense (5'-ATA CCT CCG CCG GTG ACC-3') and antisense (5'-AGT CCG CGT ATC CAC AAA GC-3'); aggrecan sense (5'-AGA GCGCGTCTAGAAATCG-3') and antisense (5'-AGC ATC AGT CGG GGA GGT G-3'); and L30, as the housekeeping gene [36], sense (5'-AGG AAG GCT CAA CGA GAA CA-3') and antisense (5'-CGA GGA GCA GAA ACC TTC AC-3'). Normalized gene expression was determined by [37]:

$$\text{Normalized Expression} = \frac{E_{\text{GOI}}^{\Delta C_{T(\text{GOI})}(\text{calibrator-sample})}}{E_{\text{HKG}}^{\Delta C_{T(\text{HKG})}(\text{calibrator-sample})}}$$

where E is the efficiency of the gene amplification, GOI is the gene of interest, HKG is the housekeeping gene (L30), and C_T is the cycle threshold point. To assess the effects of RGD as a biochemical cue in the absence of loading, control PEG-RGD hydrogels were normalized by control PEG-only gels (i.e., calibrator). To assess the combined effects of loading and RGD, loaded PEG-RGD gels were normalized to control PEG-only gels (i.e., calibrator). To isolate the effects of RGD as a biomechanical cue, loaded PEG or PEG-RGD gels were normalized to their control counterparts (i.e., calibrator). A sample size of 3 was used.

2.9 Cell Metabolism

After 48 hours in the bioreactor, control and dynamically loaded constructs were removed, crushed using a tissue homogenizer and enzymatically digested (125µg/mL of papain (Worthington Biochemical, New Jersey, USA), 10mM of L-cysteine-HCl (Sigma, Missouri, USA), 100mM of phosphate (Sigma) and 10mM of EDTA (Biorad, California, USA), pH of 6.3) for 16 hours at 60°C. Hoeschst 33258 (Polysciences, Pennsylvania, USA) assay was used to quantify total DNA content in the papain digests [38]. The amount of [³H] thymidine incorporation into newly synthesized DNA was determined in the papain digests by precipitation in trichloroacetic acid [39]. The amount of ³⁵SO₄²⁻ incorporation into newly synthesized proteoglycans was determined in the papain digests using the Alcian blue precipitation method [39]. Hydroxyproline was measured as an indicator of total collagen content in the digests [40]. Cell proliferation, proteoglycan synthesis and total collagen content were normalized to total DNA content. A sample size of 6 was used.

2.10 Statistical Analysis

Data are reported as mean values ± standard deviation. Single factor ANOVA was used and a confidence level of 0.95 was considered significant.

3. Results

3.1 Macroscopic properties for acellular and cell-laden hydrogel constructs

To determine whether the presence of RGD influenced the gel macroscopic properties, equilibrium swelling properties and tangent compressive moduli were examined for the PEG-only hydrogels and PEG-RGD hydrogels at 0.1, 0.4, and 0.8 mM RGD. The macroscopic properties were evaluated in the absence of cells and the findings are given in Table 1. No significant differences in the compressive moduli or the equilibrium swelling properties were found between the PEG-only and PEG-RGD gels and as a function of RGD concentration.

The mechanical properties of the cell-laden constructs were also assessed throughout the experiment to assess whether the loading environment impacted the construct properties. The tangent compressive moduli data are given in Table 2. No significant differences in the compressive moduli were found between the PEG-only and PEG-RGD gels at each time point

and for each culture condition (control and loaded specimen) as well as a function of RGD concentration.

3.2 Role of RGD on cell morphology in the absence and presence of an applied strain

The role of RGD on cell morphology was assessed for chondrocytes encapsulated in PEG-only gels and in PEG-RGD gels. Cell morphology was quantitatively assessed by measuring a diameter ratio, where a value of one indicates a spherical cell. Initially, chondrocytes encapsulated within these hydrogels were examined in the absence of an applied strain to determine if the presence of RGD impacted cell morphology. After the initial 24 hour free swelling period, the diameter ratios were similar in all constructs, PEG-only gels and PEG-RGD gels, (Figure 1b), with no evidence of cell spreading in the RGD containing gels as seen in the representative images of live cells encapsulated in the hydrogels (Figure 1c).

After the initial 24 hour free swelling period, the hydrogel constructs were placed into a custom designed bioreactor for 48 hours where a set of gels were subjected to dynamic loading and another set served as controls where impermeable loading platens simply rested on the gel, but did not experience any dynamic loading (see Figure 1a). Because inhibition of nutrient exchange by the impermeable platen may influence cellular response, the experimental set-up control was selected in an effort to minimize this contribution on chondrocyte function in response to dynamic loading.

We assessed cell morphology after the 48 hour culture period in the bioreactor for both the control and dynamically loaded specimens. The diameter ratios for the control specimen are given in Figure 2a. In the control PEG-only hydrogels at 0% strain, the diameter ratios were unity indicating the cells retained their rounded morphology despite being placed under impermeable pins. However, the presence of RGD in the control gels led to significant decreases in the diameter ratios when compared to PEG-only gels ($p < 0.001$). Representative images of cell morphology are shown in the upper panels of Figure 2d. The images show a relatively uniform deformation of the cells in the direction of the resting platen suggesting that the weight of the platen influenced cell morphology and led to cell deformation within the PEG-RGD gels. No additional differences in the diameter ratios were observed as a function of RGD concentration.

Post-loading, cell morphology was assessed for the loaded specimens at 0% strain and 18% strain. At 0% strain, the findings were similar to the control specimens where the diameter ratios were unity for the PEG-only gels, but significantly reduced for the PEG-RGD gels (Figure 2b). Representative images of live cells in the dynamically loaded samples at 0% strain also show similar morphologies as the control specimen (Figure 2d, middle panels). The application of an 18% compressive strain led to significant decreases in the diameter ratios compared to 0% strain conditions indicating that the cells deformed under the strain. The level of cell deformation was higher in the PEG-RGD gels compared to the PEG-only gels ($p < 0.001$ for all RGD concentrations), but was not significantly different as a function of RGD concentration. This increase in cell deformation can also be observed in the representative micrographs of chondrocytes within strained hydrogels (Figure 2d, lower panel).

Six hours post-loading, cell morphology was assessed for the control and loaded specimen at 0% strain (Figure 2c). Diameter ratios were unity for the PEG-only and PEG-RGD gels, which were previously cultured under control or loaded conditions. This finding indicates that the encapsulated chondrocytes re-adopted their rounded morphology after equilibrating to a free swelling environment.

3.3 Role of RGD on gene expression, cell proliferation and matrix production

To elucidate the effects of RGD incorporation on gene expression, aggrecan (Figure 3a), collagen type II (Figure 3b), and collagen type I (Figure 3c) expressions by chondrocytes encapsulated in PEG-RGD gels were normalized to the expression levels for PEG-only gels for the control treatment group. Incorporation of RGD into the PEG network did not significantly affect aggrecan expression. Collagen type II expression increased significantly with 0.1 mM RGD ($p < 0.05$). However, higher concentrations of RGD (0.8 mM) resulted in a significant inhibition in collagen type II expression compared to PEG-only gels and gels containing 0.1 and 0.4 mM RGD. Collagen type I expression significantly increased as RGD concentration increased ($p < 0.05$).

To assess chondrocyte phenotype, a differentiation index defined as the ratio of collagen type II/collagen type I expression (Figure 3d) was employed. The differentiation index for PEG-only gels was approximately 14 and remained similarly high for PEG-RGD gels containing 0.1 mM or 0.4 mM RGD. However, the 0.8 mM RGD gels resulted in a 75–80% decrease in the differentiation index compared to all other gel conditions.

Cell proliferation was assessed by [^3H]-thymidine incorporation into newly synthesized DNA while extracellular matrix production was quantified by $^{35}\text{SO}_4^{2-}$ incorporation into newly synthesized proteoglycans and by hydroxyproline content as a measure of total collagen content for PEG-only and PEG-RGD gels. To assess the effects of RGD on chondrocyte function, the mean response from the PEG-RGD control gels was normalized to that of the PEG-only control gels. The results are shown in Figure 4. The incorporation of RGD did not influence cell proliferation (Figure 4a), proteoglycan synthesis (Figure 4b), or total collagen production (Figure 4c).

3.4 Role of RGD and dynamic loading on gene expression, cell proliferation and matrix production

To assess the combined effects of RGD and dynamic loading, gene expression for each of the loaded hydrogels was normalized to the PEG-only controls. Normalized aggrecan, collagen type II, and collagen type I expressions are shown in Figure 5. No changes were found in aggrecan expression as a function of dynamic loading and with the addition of RGD. Collagen type II expression increased significantly with 0.1 mM RGD compared to PEG-only gels, but the expression decreased with increasing RGD concentrations ($p < 0.01$). Collagen type I expression was highest in gels containing 0.1 mM RGD. Further increases in RGD concentration did not have any additional effects on collagen type I expression. The differentiation index for the PEG-only gels was ~7 and did not change in gels with 0.1 and 0.8 mM RGD. However, the differentiation index was ~50% higher in the 0.4 mM RGD hydrogels.

To assess the combined effects of RGD and dynamic loading on cell metabolism, the mean response for cell proliferation (Figure 6a), proteoglycan synthesis (Figure 6b) and total collagen content (Figure 6c) in the dynamically loaded gels was normalized to that of the PEG-only control gels. No changes in cell proliferation were found between the PEG-only and PEG-RGD gels. However, a significant decrease in cell proliferation was observed as RGD concentration increased from 0.1 mM to 0.8 mM. No changes in proteoglycan synthesis were found between the PEG-only and PEG-RGD gels at 0.1 mM, 0.4 mM and 0.8 mM. However, compared to 0.4 mM RGD the other PEG-RGD gels showed a significant increase in proteoglycan synthesis. Total collagen content significantly decreased as RGD concentration increased to 0.1 mM. Further increases in RGD concentration from 0.1 to 0.8 mM led to significant increases in collagen content which reached values similar to the PEG-only gels.

3.5 Role of RGD as a mechanotransducer on chondrocyte gene expression, cell proliferation and matrix production

In an effort to isolate the role of RGD as a biomechanical signal and its potential involvement in integrin-mediated mechanotransduction, the mean cellular responses from the dynamically loaded PEG gels were normalized to their control counterparts. The normalized cellular responses for gene expression, cell proliferation, and matrix production are given in Figures 7 and 8.

Aggrecan expression was similar in the PEG-only gels compared to each of the PEG-RGD gels. However, a higher normalized aggrecan expression (Figure 7a) was present in the 0.8 mM RGD gels compared to the 0.1 mM RGD gels ($p < 0.01$). Collagen type II expression (Figure 7b) was higher in the 0.1 mM RGD and even higher in the 0.8 mM RGD gels compared to the PEG-only gels. However, collagen type II expression in the 0.4 mM RGD gels was similar to the PEG-only gels. Collagen type I expression (Figure 7c) was highest in gels containing 0.1 mM RGD but as RGD concentration increased from 0.1 mM to 0.8 mM collagen type I expression decreased significantly ($p < 0.001$). The normalized differentiation index increased significantly with increasing concentrations of RGD ($p < 0.05$).

Cell proliferation decreased with increasing RGD concentrations and was inhibited by ~50% in the 0.8 mM RGD hydrogel specimen compared to PEG-only and 0.1 mM RGD gels (Figure 8a; $p < 0.05$). Proteoglycan synthesis (Figure 8b) was highest at 0.1 mM RGD compared to the PEG-only and PEG-RGD gels containing higher RGD concentrations ($p < 0.05$). Total collagen content (Figure 8c) decreased significantly with 0.1 mM RGD compared to PEG-only gels ($p < 0.05$), but increased with higher RGD concentrations ($p < 0.05$) to values that were statistically similar to the PEG-only gels.

4. Discussion

Mechanical loading produces complex changes within the tissue leading to extracellular events such as cell deformation, fluid flow, and streaming potentials, which may influence cellular response through a variety of mechanotransduction pathways [1,9,10,14,41–43]. Mechanocoupling of the cell to its surrounding pericellular matrix is another mechanism by which chondrocytes may sense mechanical force (e.g., via integrin-mediated mechanotransduction) [1,5,16,17]. In cartilage, these mechanisms are highly inter-related making it difficult to isolate the different extracellular events and study their involvement in chondrocyte mechanotransduction. Our goal for this study was to employ a 3D culture system based on PEG where PEG does not permit cell interactions, but to which matrix analogs (i.e., RGD ligands) can be introduced in a highly controlled manner. Therefore, PEG-only gels served as a base 3D environment to study extracellular events associated with dynamic loading in the absence of any cellular mechanocoupling to the hydrogel. The incorporation of RGD into the PEG hydrogel facilitates chondrocyte interaction with the hydrogel allowing us to explore the role of RGD as a biochemical and biomechanical cue.

Characterization of the PEG-only and PEG-RGD hydrogels revealed similar swelling behavior, compressive moduli, and initial cell morphologies indicating that the extracellular events that occur during dynamic loading such as fluid flow and cell deformation are initially similar in all gel formulations. However, upon removal of the constructs from the bioreactor after 48 hours, cell morphology was very different and dependent on the presence of RGD. Specifically, we observed that the chondrocytes in the PEG-RGD constructs that were either subjected to small static strains imparted by the loading platen in the control specimen or subjected to dynamic loading retained an oblate ellipsoidal morphology immediately upon removal from the bioreactor. This phenomenon was not observed in the PEG-only constructs, where chondrocytes re-adopted a spherical morphology once removed from the bioreactor.

Interestingly, the gross mechanical properties were similar for the PEG-only and PEG-RGD hydrogels throughout the experiment suggesting that the repetitive strains applied to the control or loaded specimen did not impact the material properties. The integrin-ligand interaction, however, is a dynamic process with formation and dissolution of focal adhesions, such as seen during cell migration [44]. Several studies have shown that mechanical forces lead to increased adhesion strength of the integrin-ligand bond, which increases with higher ligand concentration [45,46]. Therefore, it is possible that the combination of mechanical stimulation and interaction with RGD facilitated stronger interaction of the cells with the hydrogel resulting in the more deformed morphology as seen by confocal microscopy (Fig 2d). However, this effect was only temporary and the cells eventually re-adopted their original spherical morphology once the strain was removed long-term further suggesting a dynamic interaction between the tethered RGD ligands and the chondrocytes. However, additional studies are necessary to elucidate the role of RGD in mediating changes in cellular morphology as a result of applied strains.

In the absence of an applied dynamic compressive strain, the presence of RGD did not appear to affect the chondrocyte phenotype at the lower RGD concentrations. However, at the highest concentration of RGD employed in this study (0.8 mM), chondrocyte phenotype, as measured by the differentiation index, was dramatically inhibited suggesting that chondrocytes are dedifferentiating. It is well known that chondrocytes have the capacity to dedifferentiate into a fibroblast-like phenotype, typically identified by a decrease in collagen type II expression and an increase in collagen type I expression [47–49]. Alsberg *et al.* [6] demonstrated that immobilizing RGD to alginate hydrogels, at concentrations similar to our lowest concentration, significantly enhanced *in vivo* cartilaginous tissue formation by encapsulated chondrocytes over alginate hydrogels without RGD. However, other studies have demonstrated that the presence of immobilized RGD tethers inhibits chondrogenesis of bovine derived mesenchymal stem cells in 3D alginate [50] and agarose [51] hydrogels. Although, Salinas *et al.* [30] found that RGD improves chondrogenesis for adult human mesenchymal stem cells in 3D PEG hydrogels containing RGD tethers. Taken together, these findings indicate that chondrocytes and chondrogenesis of mesenchymal stem cells are sensitive to integrin-mediated events involving RGD, but the effect of RGD appears to be dependent on the concentration, culture system, and cell source. Interestingly, RGD at the concentrations studied here had no effect on matrix production or cell proliferation. However, our studies were limited to short culture times of 48 hours. Longer culture times may be needed to detect changes in cellular response due to the presence of RGD, but in the absence of dynamic loading.

The mechanical loading environment associated with the 3D PEG hydrogel environment (i.e., dynamically loaded PEG-only gels normalized to their respective controls, Figures 7, 8) did not influence ECM gene expression, cell proliferation or proteoglycan synthesis, but resulted in a 2-fold increase in collagen deposition. This finding suggests that the extracellular environment associated with fluid flow and/or cell deformation mediates collagen deposition. Previous studies have reported that fluid flow enhances overall collagen deposition, and specifically collagen type II, in 3D engineered cartilage tissues [52]. The effects of cellular deformation on chondrocyte function are less understood, in part, due to the difficulty in decoupling the effects of dynamic cellular strains with other extracellular events such as fluid flow.

Investigation into the mechanical role of RGD indicates that cellular responses are dramatically different for the mechanically stimulated constructs compared to the control constructs. When chondrocytes interact with RGD in the presence of dynamic loading, the higher concentrations of RGD enhance the chondrocyte phenotype, as observed by an increase in the differentiation index. This finding is contrary to our results where in the absence of loading the high RGD concentration negatively impacted chondrocyte phenotype. Furthermore, the presence of RGD influenced cell proliferation, proteoglycan synthesis, and collagen deposition, but only when

loading was applied. This further suggests a very different role for RGD involved in mechanotransduction events compared to RGD acting a biochemical signal. For example, RGD acting as a mechanotransducer had a positive effect on proteoglycan synthesis, particularly for the 0.1 mM RGD concentration, while RGD in the absence of loading had no effect on matrix synthesis. Interestingly, collagen production was inhibited at the lower RGD concentrations when compared to the PEG-only hydrogels, even though collagen type II expression was 3-fold higher. Although, collagen production represents the total collagen produced and does not provide insight into the type of collagen being deposited. Nonetheless, with increasing RGD concentration, collagen production increased. It is also important to note that a scrambled sequence, RDG, tethered to the PEG hydrogel, had no effect on chondrocyte response to mechanical loading (unpublished data). Together, these findings indicate chondrocytes are responsive to RGD when mechanically stimulated suggesting an important role for RGD in chondrocyte mechanosensing.

However, it is also important to consider the effects of cellular deformation, which at the end of the experiment were significantly greater in the RGD containing gels compared to the PEG-only gels. The application of large strains has been shown to have an inhibitory effect on matrix biosynthesis by the chondrocyte [53]. Interestingly, the level of cell deformation was statistically similar for all three RGD concentrations suggesting that the difference in responses observed in the PEG-RGD gels are likely due to integrin-mediated mechanotransduction events.

One potential limitation of our study is due to the fact that chondrocytes are encapsulated in PEG gels containing RGD and cultured under free swelling conditions for 24 hours prior to being subjected to dynamic loading. It is possible that this initial culture period may have led to differences in matrix deposition, which in turn influenced cell response. Although, we did not observe differences in collagen production or proteoglycan synthesis in the control gels after the 72 hour culture period, it is possible that the presence of RGD could have influenced the type of collagen deposited and/or other ECM proteins (e.g., fibronectin) in the initial 24 hour culture period. Therefore, our findings may be due to an indirect response to RGD where any difference in the PCM composition can alter the biochemical or biomechanical signals perceived by the chondrocyte and hence their metabolic activities [1,54,55]. Additional studies that probe chondrocyte response when cells are isolated with their intact pericellular matrix, i.e., chondrons, may help to elucidate the role of PCM in mediating chondrocyte response. It is also important to note that the concentrations of RGD employed in this study are likely below the saturation limits of available RGD attachment sites allowing for newly deposited proteins such as fibronectin to interact with the chondrocyte through the RGD sites. Additional studies are needed which span a wider range of RGD concentrations towards isolating the specific RGD-integrin interactions involved in chondrocyte mechanotransduction.

5. Conclusion

Our findings demonstrate that chondrocyte response to RGD is dramatically different in the absence and presence of mechanical stimulation. In the absence of dynamic mechanical stimulation, the presence of RGD appears to have a negative effect on chondrocyte phenotype and little effect on early chondrocyte bioactivity (i.e., cell proliferation and matrix synthesis/deposition). In the presence of dynamic mechanical stimulation, RGD appears to act as a mechanotransducer showing improved chondrocyte phenotype and proteoglycan synthesis. Interestingly, we observed that the level of cell deformation resulting from the application of a mechanical strain is influenced by chondrocyte mechanocoupling to the hydrogel via the RGD ligands. This finding suggests that integrins may mediate chondrocyte morphology in response to mechanical strain. Although the exact mechanism involved in integrin-mediated mechanotransduction are still to be elucidated, this study demonstrates an important finding

where the incorporation of tethered cell adhesion moieties, specifically RGD, enhances the chondrocyte phenotype and matrix synthesis, but only when dynamic stimulation is employed. Overall, our findings further support evidence in the literature that cell-matrix interactions, in part, mediate chondrocyte response to mechanical stimulation. Studies are underway by the authors where we are building upon our current study to investigate in more detail the mechanisms involved in integrin-mediated mechanotransduction events associated with chondrocyte interaction with RGD.

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Figure 1.

(a) Schematic describing the experimental design where hydrogel constructs were cultured under free swelling conditions for 24 hours and then placed into the bioreactor as either controls (i.e., cultured under the loading platen, but not subjected to dynamic loading) or as dynamically strained constructs (0.3 Hz with amplitude strains of 18%). (b) Chondrocyte morphology as a function of hydrogel chemistry after the 24 hour initial free swelling period. Morphology was quantified by a diameter ratio (x/y) defined by the ratio of the minimum cell diameter (x) to the maximum cell diameter (y), which was measured at full width half maximum height. (c) Representative micrographs of chondrocytes encapsulated in PEG and PEG-RGD hydrogels after free swelling for 24 hours. Original magnification is 40 $\times$ oil.

Figure 2.

(a) Chondrocyte morphology in the control constructs after being cultured in the bioreactor for 48 hours. Morphology was quantified by a diameter ratio (x/y) defined by the ratio of the minimum cell diameter (x) to the maximum cell diameter (y), measured at full width half maximum height. (b) Chondrocyte morphology in the dynamically loaded constructs after being cultured in the bioreactor for 48 hours. To represent cell morphology during mechanical stimulation, diameter ratios were assessed at 0% strain (black bars) and 18% strain (gray bars). Diameter ratios (x/y) were defined by the ratio of the cell diameter parallel to direction of applied strain (x) to the cell diameter perpendicular to the direction of applied strain (y). (c) Chondrocyte morphology in the control (black bar) and dynamically loaded constructs (gray bar) 6 hours after removal from the bioreactor. Morphology was quantified by the ratio of the minimum cell diameter (x) to the maximum cell diameter (y), measured at full width half maximum height. (d) Representative micrographs of chondrocytes encapsulated in PEG and

PEG-RGD hydrogels controls or dynamically strained constructs immediately after removal from the bioreactor. Original magnification is 40× oil.

Figure 3.

Normalized gene expression for aggrecan (a), collagen type II (b), and collagen type I (c). (d) Chondrocyte phenotype was assessed by a differentiation index defined by the ratio of collagen II to collagen I expressions. Relative gene expression for the control gels was normalized to the relative gene expression of the 0 mM RGD control gel to assess the effects of RGD as a biochemical cue. Data are given by mean $\pm$ standard deviation (n=3); *p<0.05, **p<0.01, ***p<0.001.

Figure 4.

Normalized cell proliferation (a), proteoglycan synthesis (b) and collagen content (c) for control gels. Data were normalized to the 0 mM RGD control gels to assess the effects of RGD as a biochemical cue. Data are given by mean $\pm$ standard deviation (n=6); *p<0.05, **p<0.01, ***p<0.001.

Figure 5. Normalized gene expression for aggrecan (a), collagen type II (b), and collagen type I (c). (d) Chondrocyte phenotype was assessed by a differentiation index defined by the ratio of collagen II to collagen I expressions. Relative gene expression for the dynamically strained constructs were normalized to the relative gene expression of the 0 mM RGD control gel to assess the combined effects of RGD as a biochemical and biomechanical cue. Data is given by mean $\pm$ standard deviation (n=3); *p<0.05, **p<0.01, ***p<0.001.

Figure 6.

Normalized cell proliferation (a), proteoglycan synthesis (b) and collagen content (c) for dynamically strained gels. Data for the dynamically strained gels were normalized to the 0 mM RGD control gels to assess the combined effects of RGD as a biochemical and biomechanical cue. Data are given by mean $\pm$ standard deviation (n=6); *p<0.05, **p<0.01, ***p<0.001.

Figure 7.

Normalized gene expression for aggrecan (a), collagen type II (b), and collagen type I (c). (d) Chondrocyte phenotype was assessed by a differentiation index defined by the ratio of collagen II to collagen I expressions. Relative gene expressions for the dynamically strained constructs were normalized to the relative gene expression the respective control gels to assess the role of RGD as a biomechanical cue. Data is given by mean $\pm$ standard deviation (n=3); *p<0.05, **p<0.01, ***p<0.001.

Figure 8.

Normalized cell proliferation (a), proteoglycan synthesis (b) and collagen content (c) for dynamically strained gels. Data for the dynamically strained gels were normalized to their respective control gels to assess the role of RGD as a biomechanical cue. Data are given by mean $\pm$ standard deviation (n=6); *p<0.05, **p<0.01, ***p<0.001.

Table I

Macroscopic Properties of PEG and PEG-RGD hydrogels*

RGD Concentration, mM	0	0.1	0.4	0.8
Compressive Modulus (kPa)	55 ± 21	62 ± 11	75 ± 8	57 ± 12
Volumetric Equilibrium Swelling Ratio	11 ± 0.04	11 ± 0.2	11 ± 1	11 ± 0.1

* Properties were measured in cell-free hydrogels

Table II

Mechanical Properties of Cell-laden PEG-only and PEG-RGD Hydrogels

RGD Concentration, mM	Compressive Modulus (kPa)		
	24 hours	72 hours	
	Free Swell	Control	Loaded
0.0	59 ± 16	64 ± 6	55 ± 9
0.1	58 ± 11	58 ± 14	49 ± 4
0.4	55 ± 8	51 ± 4	62 ± 19
0.8	62 ± 6	52 ± 10	47 ± 2