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Biomaterials 26 (2005) 5991–5998
Biomaterials
www.elsevier.com/locate/biomaterials

The effect of incorporating RGD adhesive peptide in polyethylene glycol diacrylate hydrogel on osteogenesis of bone marrow stromal cells

Fan Yang, Christopher G. Williams, Dong-an Wang, Hyukjin Lee, Paul N. Manson, Jennifer Elisseeff*

Department of Biomedical Engineering, Johns Hopkins University, 3400 North Charles Street/Clark Hall 106, Baltimore, MD 21218, USA

Received 3 December 2004; accepted 7 March 2005
Available online 4 May 2005

Abstract

Advances in tissue engineering require biofunctional scaffolds that can not only provide cells with structural support, but also interact with cells in a biological manner. To achieve this goal, a frequently used cell adhesion peptide Arg–Gly–Asp (RGD) was covalently incorporated into poly(ethylene glycol) diacrylate (PEODA) hydrogel and its dosage effect (0.025, 1.25 and 2.5 mM) on osteogenesis of marrow stromal cells in a three-dimensional environment was examined. Expression of bone-related markers, osteocalcin (OCN) and Alkaline phosphatase (ALP), increased significantly as the RGD concentration increased. Compared with no RGD, 2.5 mM RGD group showed a 1344% increase in ALP production and a 277% increase in OCN accumulation in the medium. RGD helped MSCs maintain cbfa-1 expression when shifted from a two-dimensional environment to a three-dimensional environment. Soluble RGD was found to completely block the mineralization of marrow stromal cells, as manifested by quantitative calcium assay, phosphorus elemental analysis and Von Kossa staining. In conclusion, we have demonstrated that RGD-conjugated PEODA hydrogel promotes the osteogenesis of MSCs in a dosage-dependent manner, with 2.5 mM being optimal concentration.

Keywords: RGD; Marrow stromal cells; Osteogenesis; Photopolymerizing; Hydrogel

1. Introduction

Bone loss and nonunion defects caused by traumatic events, congenital abnormalities and diseases have been conventionally treated using autograft and allograft of bone tissues [1]. These approaches can be very successful but are limited by procurement difficulties, insufficient material supply, donor site morbidity and contour irregularities. Recent progress in the field of tissue engineering may provide an alternative approach to aid in bone repair by creating a microenvironment that could induce transplanted cells residing in a biomedical scaffold to produce a desired extracellular matrix and thus regenerate bone tissues.

Advances in tissue repair require biofunctional materials that not only give cells structural support, but also interact with cells to promote desired biological functions. Extensive studies have incorporated various biological signals into scaffolds via surface coating or bulk modification and have evaluated their effects on cell behavior such as adhesion, spreading, proliferation and matrix production. The cell adhesion peptide, Arg–Gly–Asp (RGD), resides in the cell attachment region of fibronectin and has been intensively studied as a cell-binding sequence. Mann et al. [2] examined the effects of surface RGD density on the ECM production of smooth muscle cells, endothelial cells and fibroblasts and showed that all these cell types exhibited decreased

*Corresponding author. Tel.: 410 516 4915; fax: 410 516 8152.
E-mail address: jhe@bme.jhu.edu (J. Elisseeff).

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doi:10.1016/j.biomaterials.2005.03.018

ECM production on the more adhesive surface. Rowley et al. [3] tested myoblasts on an RGD-coated alginate hydrogel surface and demonstrated that RGD can promote cell adhesion, spreading and proliferation in a dose-dependent manner. These effects were also seen in a three-dimensional environment when RGD was incorporated into the scaffold via bulk modification. Schmedlen et al. [4] demonstrated that RGD modified poly vinyl alcohol (PVA) supported the attachment and spreading of fibroblasts as the RGD concentration increased. Burdick et al. [5] tested the effect of Acr–PEG–RGD on rat calvarial osteoblasts and demonstrated increased osteoblast attachment, spreading and mineralization in a dose-dependent manner. Shin et al. [6] modified oligo [poly(ethylene glycol) fumarate] (OPF) hydrogels with GRGD and a PEG spacer. Hydrogels made of OPF 1.0 K and 3.3 K modified with GRGD peptide allowed rat MSC attachment and spreading 24 h after seeding. RGD peptide facilitated cell adhesion and spreading in both two-dimensional and three-dimensional environments in all these studies.

Hydrogel is a genre of scaffold that is widely used in tissue engineering applications due to its high tissue-like water content, ability to homogeneously encapsulate cells, efficient mass transfer and easily manipulatable physical properties [7]. Photopolymerization is a technique that employs light to generate radicals from photoinitiators, which will further react with the active end group on polymers to form a covalent crosslink. Compared with other approaches, photopolymerized hydrogel systems provide better temporal and spatial control over the gelation process, are injectable in nature, and can polymerize in situ to fill defects of any shape. Poly(ethylene-glycol)-diacrylate (PEGDA) is a promising tissue engineering scaffold candidate for such applications. Previous studies have demonstrated that PEGDA can be used to photoencapsulate chondrocytes and marrow stromal cells (MSCs) to form a cartilage-like tissue [8,9]. However, for bone tissue engineering purposes, poly(ethylene glycol), PEG, is notable for its lack of adhesiveness while adhesion and spreading facilitates osteoblasts matrix deposition during bone remodeling [12].

Although effects of RGD on cell adhesion and spreading have been extensively studied, it is not until recently that its effect on cell differentiation was examined. For bone tissue engineering applications, it has been shown that surface modification with RGD-containing peptide can promote osteoblast differentiation [13–18]. Bone marrow derived stromal cells (MSCs) have gained tremendous attention as an autologous or allogeneic cell source that can be easily isolated, are highly proliferative and capable of differentiating down multiple lineages including chondrocytes, osteoblasts, adipocytes, neurons, etc [10,11]. For MSC-based bone tissue engineering, we hypothesize that increasing cell adhesion and spreading will promote the differentiation of MSCs into osteoblast-like cells under standard osteogenic conditions. The overall goal of this study is to evaluate the effect of RGD mediated cell adhesion on osteogenesis of MSCs in a photopolymerizing PEGDA hydrogel by examining bone-specific protein secretion, mineralization and gene expression. Specifically, we aim to answer the following questions: (1) does RGD-conjugation to a hydrogel promote the osteogenic differentiation of MSCs? (2) do MSCs differentiate in a RGD dosage-dependent manner? (3) how would soluble RGD saturation affect MSC osteogenesis in an RGD-conjugated hydrogel? The results presented below specifically address these questions.

2. Materials and methods

2.1. Peptide conjugation to PEG

YRGDS was reacted with acryloyl-PEG-*N*-hydroxy-succinimide (acryloyl-PEG-NHS, 3400 Da; Nektar Therapeutics, Huntsville, AL) in a 1:2.6 molar ratio in 50 mM TRIS buffer (pH 8.2) for 2 h at room temperature. Excessive acryloyl-PEG-NHS was used to assure full reaction of the peptide. The product was then lyophilized and stored at −20 °C.

2.2. MSC isolation and expansion

Marrow stromal cells (MSCs) were isolated from goat bone marrow as previously reported [8]. Cells were plated on 175 cm^2 tissue culture flasks at an initial density of approximately 120,000 mononuclear cells/cm^2 as determined by hemocytometer. The first medium change occurred after 4 days to allow cell attachment and three times a week thereafter until confluency (12–14 days). Cells were passaged with 0.025% trypsin–EDTA (Clonetics Biowhittaker, Walkersville, MD). Passage 3 cells were trypsinized, centrifuged and resuspended in the hydrogel solution.

2.3. Hydrogel encapsulation

Polymer was prepared by mixing poly(ethylene glycol)-diacrylate (PEGDA; Nektar Therapeutics, Huntsville, AL) and YRGDS-PEG-Acrylate in sterile phosphate-buffered saline (PBS) with penicillin (100 U/ml) and streptomycin (100 μg/ml) (GIBCO Invitrogen, Carlsbad, CA) to make a 15% (w/v) hydrogel. Three different concentrations of RGD were tested: 0.025, 1.25, and 2.5 mM. PEGDA hydrogel (15% (w/v)) with no RGD served as a control. Photoinitiator, Igracure D2959 (Ciba Speciality Chemicals, Tarrytown, NY), was added to the hydrogel solution and mixed thoroughly to make a final concentration of 0.05% (w/

v). Passage 3 MSCs were homogeneously suspended in the hydrogel solution to make a concentration of 15 million cells/ml. Seventy-five microliters of the cell–polymer mixture were loaded into cylindrical molds (6 mm in diameter), and exposed to UV light (365 nm, 4 mW/cm^2) for 5 min to achieve gelation. The hydrogels were removed from the molds, washed with sterile PBS, and transferred to 12-well culture plates with osteogenic medium.

2.4. Saturation of cell surface RGD receptors with soluble RGD

Soluble RGD was used to saturate the cell surface RGD receptors and inhibited MSC attachment to peptide modified hydrogel. Soluble RGD peptide (Sigma Aldrich, A-8052) was added to MSCGM in a concentration of 0.5 mM and incubated with MSCs for 30 min, with periodic mixing every 10 min. The pretreated MSCs were then encapsulated in a PEGDA/YRGDS-PEG-Acrylate cogel that contained 2.5 mM RGD following the encapsulation procedure described above. Soluble RGD (0.5 mM) was also added in the culture medium throughout the 3-week culture period. Two control groups were 2.5 mM RGD cogel with and without MSCs, both without soluble RGD incubation.

2.5. In vitro cultivation

The hydrogel samples were all incubated at 37 °C in 5% CO_2 in 2 ml of osteogenic medium. Osteogenic medium consists of high-glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO), 100 nM dexamethasone (Sigma), 50 μg/ml ascorbic acid 2-phosphate (Sigma), 10 mM β-glycerophosphate (Sigma), 10% fetal bovine serum (Gibco), 100 unit/ml penicillin and 100 μg/ml streptomycin (Gibco). Medium was changed every 2–3 days.

2.6. Biochemical assays

Hydrogels were moved to serum-free DMEM culture medium 24 h before harvest to remove the serum components trapped inside the gel. The samples were then harvested for quantitative osteocalcin (OCN) ELISA, alkaline phosphatase, calcium and DNA assays and the medium was also collected for OCN and ALP assays ($n = 3$/group). The OCN ELISA assay was performed with Metra Osteocalcin EIA Kits (Cat 8002, Quidel, San Diego, CA) following manufacturer's instruction. For quantitative alkaline phosphatase assay, hydrogel constructs were homogenized in 0.75 M 2-amino-2-methyl propanol (AMP, Sigma; pH 10.3) solution and the supernatants were collected for ALP assay using Sigma Alkaline Phosphatase Determination Kits (Sigma Diagnostics 245) following manufacturer's protocol. DNA assay was performed as previously described [8]. For quantitative calcium measurement, lyophilized hydrogel samples were homogenized in 0.5 M HCl and vigorously vortexed for 16 h at 4 °C. The supernatant was collected for calcium assay following manufacturer's protocol (Sigma Diagnostics 587). Wet weights and dry weights after 48 h lyophilization were obtained from all constructs for normalization of ECM contents. For the soluble RGD saturation experiment, lyophilized samples were sent for quantitative measurement of the phosphorus and carbon contents via elemental analysis (QTI Technologies).

2.7. Histology

Three constructs per group were harvested for histological evaluation at the end of three weeks. The hydrogels were fixed overnight in 4% paraformaldehyde at 4 °C and transferred to 70% ethanol until embedded in paraffin according to standard histological techniques. Sections were stained with hematoxylin and eosin for general cell morphology as well as Von Kossa for mineralization.

2.8. RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was isolated from three constructs per group and from goat MSCs of the same passage cultured in monolayer. Fresh cellular hydrogels were homogenized in Trizol (Aldrich) and RNA extraction was performed with RNeasy Minikit following manufacturer's protocol (Qiagen, Valencia, CA). RNA extraction from monolayer was directly performed with RNeasy minikit. The cDNA was synthesized by reverse transcription (RT) with Superscript First-Strand Synthesis System (Invitrogen 11904-018, Carlsbad, CA). Polymerase chain reaction (PCR) was performed using Taq DNA polymerase (Invitrogen) at annealing temperature of 60 °C (for β-actin and cbfa-1) for 35 cycles. The sequences of PCR primers (forward and backward, 5′ to 3′) were as follows: β-actin, 5′-TGGCACCACACCTTCTACAATGAGC-3′ and 5′-GCACAGCTTCTCCTTAATGTCACGC-3′; cbfa1, 5′-CCACCCGGCCGAACTGGTCC-3′ and 5′-CCTCGTCCGCTCCGGCCCACA-3′. Each PCR product was analyzed by separating 4 μl of the amplicon and 1 μl of loading buffer in 2% agarose gel in TAE buffer.

2.9. Statistical analysis

All experiments were performed in triplicate. The results were reported as mean ± standard deviation. Statistical significance was determined by analysis of variance (ANOVA single factor) and set as $P < 0.05$.

3. Results

3.1. Biochemical analyses

The DNA content was similar among all groups, indicating that tethered RGD peptide did not significantly change cell proliferation in the PEG hydrogel (Fig 1). Osteocalcin (OCN) accumulation in the last 24 h, as measured by the OCN in the medium, increased significantly as the RGD concentration increased ($P<0.02$). Compared to the 0RGD control, the three RGD-containing groups 0.025RGD, 1.25RGD, 2.5RGD accumulated 143%, 211% and 277% more OCN (Fig. 2A) respectively. Osteocalcin production within the gel was normalized to DNA content to reflect the production per cell. No statistical difference was seen in the OCN production per DNA among all the groups (Fig. 2B). Similarly, ALP production per cell also significantly increased in a RGD dosage-dependent manner ($P<0.02$). Compared with the 0RGD control, the three RGD containing groups 0.025RGD, 1.25RGD, 2.5RGD produced 77%, 357%, and 1344% more ALP (Fig. 3) respectively. The largest difference was seen in the ALP production when the RGD concentration increased from 1.25 mM to 2.5 mM.

The soluble RGD-treated group (0.0082 ± 0.0013 U/L per ww) produced a comparable amount of ALP as the control group (0.006 ± 0.0013 U/L per ww). ALP accumulation in the last 24 h was reflected by ALP in the medium. Again, no difference was observed between the soluble RGD-treated group (0.45 ± 0.04 U/L) and the control group (0.51 ± 0.26 U/L) (Fig. 4).

For the RGD-dosage experiment, there was no difference in calcium production until the RGD concentration increased to 2.5 mM. A 60% drop in the calcium content per DNA was seen in the 2.5RGD group compared to 0RGD control ($P<0.01$) (Fig. 5). For the soluble RGD saturation experiment, the control cellular group produced significant amounts of calcium (0.0268 ± 0.0057 per dw) while the soluble RGD-treated group produced almost no calcium (0.0052 ± 0.0018 per dw), which is comparable to the acellular control group (0.0041 ± 0.0010 per dw), $P<0.01$. (Fig. 6).

3.2. Elemental analysis

Carbon content per dry weight for the 2.5RGD cellular and acellular control groups, as well as the soluble RGD treated group, was 7.21%, 9.21% and 7.28%, respectively. Phosphorous elemental analysis exhibits detectable phosphorous content in 2.5RGD cellular group (0.23%) while only a minimum amount of

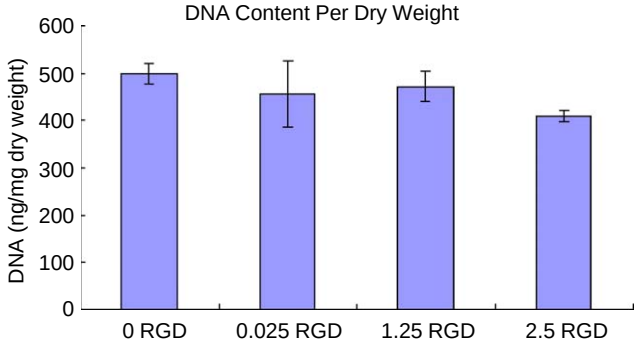


Fig. 1. The DNA content of the MSC constructs at the end of the three-weeks culture was normalized by dry weight for comparison ($n=3$).

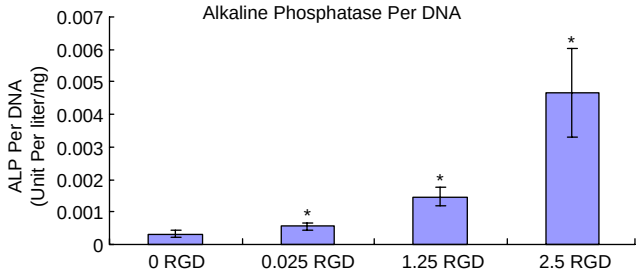
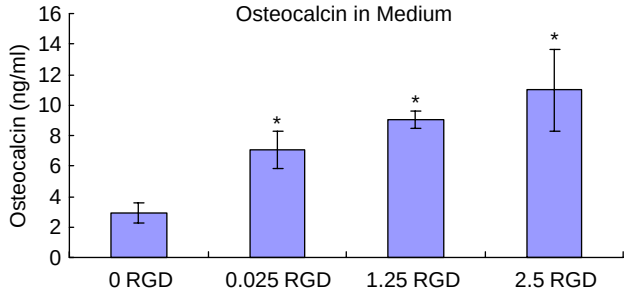


Fig. 3. Alkaline phosphatase production per cell was significantly increased in a RGD dosage-dependent manner ($n=3$, *: $P<0.02$). Compared with 0RGD control, the three RGD-containing group, produce 77%, 357% and 1344% more ALP respectively.



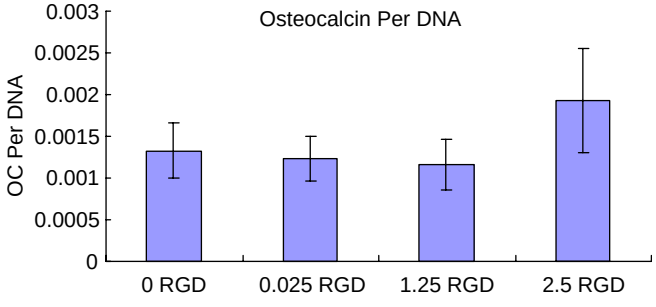


Fig. 2. Osteocalcin (OCN) accumulation in the last 24 h was measured by quantifying the OCN in the medium (left) and OCN production within the gel was normalized by DNA content to reflect the production per cell (Right). The three RGD containing group, respectively, accumulated 143%, 211% and 277% more OCN in the medium compared with 0RGD control ($n=3$, *: $P<0.02$).

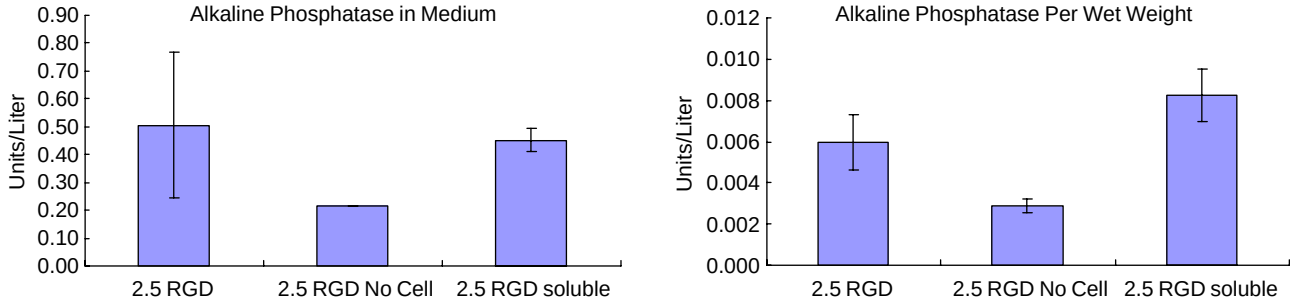


Fig. 4. For the soluble RGD saturation experiment, ALP production was quantified both in the medium (left) and per cell in the gel (right). No significant difference was seen between the soluble RGD-treated group and the control group ($n = 3$).

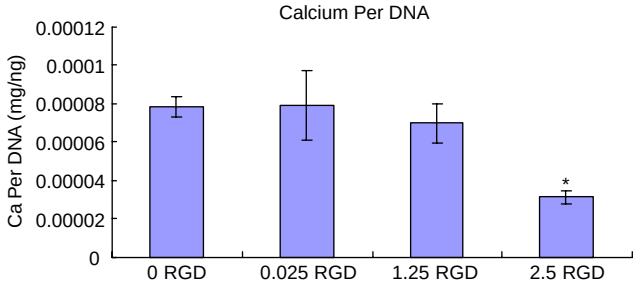


Fig. 5. For the RGD-dosage experiment, no difference was seen in the calcium production until RGD concentration increased to 2.5 mM ($n = 3$, *: $P < 0.01$).

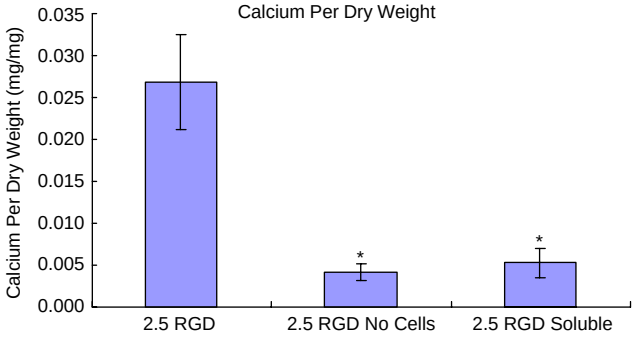


Fig. 6. For the soluble RGD saturation experiment, minimum calcium was produced in the soluble RGD-treatment group compared to nonsoluble RGD-treated group ($n = 3$,*: $P < 0.01$).

phosphorous was detected in soluble RGD-treated group and acellular control group (<0.02%). Phosphorous content was normalized by carbon content to take into account the difference in the sample size, since carbon content should be proportional to the sample size (Table 1).

3.3. Histology

Hematoxylin–eosin staining exhibited normal cell shape and morphology in both the RGD-treated group and the control cellular group. In correlation with the quantitative calcium assay data, mineralization was present in pericellular regions by von Kossa staining in the control cellular group not treated with soluble RGD. In contrast, no calcium staining was seen in either the acellular empty control gels or the experimental gels treated with soluble RGD (Fig. 7).

Table 1
Elemental analysis

	C%	P%	P/C
2.5 RGD	7.21	0.23	0.03
2.5 RGD no cell	9.71	<0.02	0
2.5 RGD soluble	7.28	<0.02	0

3.4. RT-PCR

The early osteogenesis marker, transcriptional factor cbfa-1, was expressed in the monolayer cells before encapsulation. After encapsulation, cbfa-1 expression was downregulated in the nonadhesive three-dimensional culture environment, as demonstrated by the weak cbfa-1 band in 0RGD and 0.025RGD groups. The strong expression of cbfa-1 was recovered when the RGD concentration reaches 1.25 mM or higher (Fig. 8).

4. Discussion

Previously we reported that marrow stromal cells survived photoencapsulation in PEGDA hydrogel and underwent chondrogenesis in three weeks [8]. In the present study, we conjugated the cell adhesion peptide YRGDS to PEG hydrogels and showed that this modified three-dimensional culture system can be used to promote more efficient MSCs osteogenic differentiation.

Alkaline phosphatase (ALP) is an early bone marker protein and an essential enzyme for ossification. ALP production increased with the RGD dose and its largest

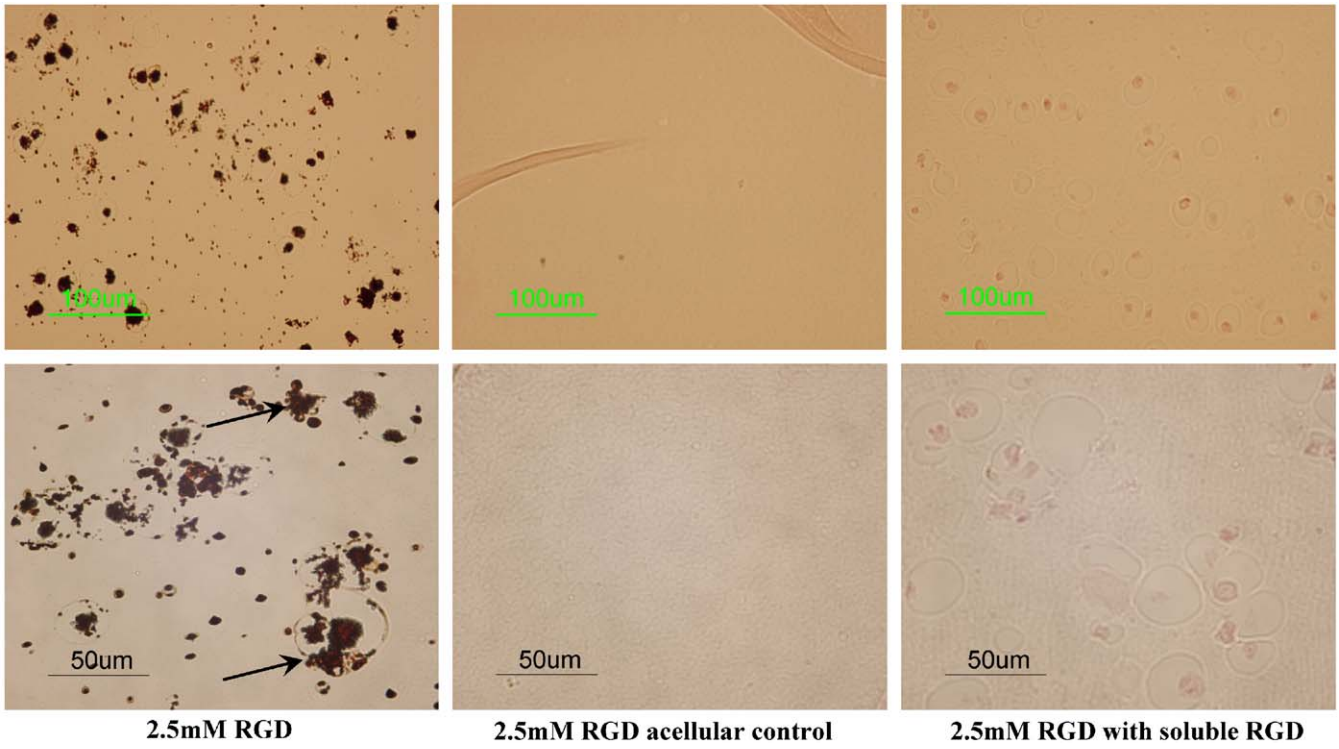


Fig. 7. Von Kossa staining demonstrated pericellular mineralization (arrows) was completely blocked by soluble RGD treatment. Top row shows results at 20 × magnification and bottom row are the same groups at a higher magnification view (40 ×) ($n = 3$).

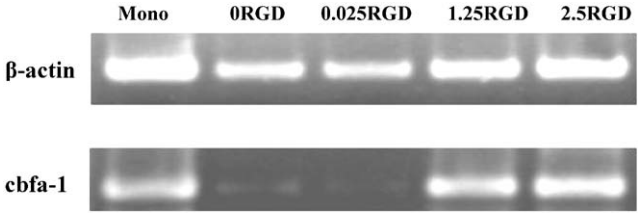


Fig. 8. Early osteogenesis marker, transcriptional factor cbfa-1 was positively expressed in the monolayer and the expression was down-regulated after encapsulation in three-dimensional hydrogel. The expression can be recovered once the RGD concentration reached 1.25 mM or higher.

jump from 1.25RGD to 2.5RGD suggests that this concentration range is a critical window for ALP regulation. We chose the scaffold tethered with the optimal RGD concentration (2.5 mM) and then examined the effect of soluble RGD saturation on ALP production. In this experiment, both soluble RGD treated and control groups employed 2.5 mM RGD tethered scaffold, thus eliminating pore size as a variable that affects tissue development [19]. Comparable amount of ALP production by the soluble RGD-treated group and the control group both within the gel and in the medium suggests that 2.5 mM is as potent as the maximal RGD concentration for stimulating ALP production.

Osteocalcin production in the RGD-dosage experiment also suggests that RGD incorporation can increase osteogenesis of marrow stromal cells in a dose-dependent manner. Osteocalcin is a small vitamin K-dependent BGP (Bone-Gla-Protein) produced by osteoblasts and regarded as a late bone marker. Detection of osteocalcin in both the medium and within the gel suggests that the encapsulated marrow stromal cells have differentiated down the osteogenic pathway and become osteoblast-like cells after three-weeks culture in the three-dimensional hydrogels. Within the gel, similar amounts of osteocalcin were detected across all concentration groups, with only 2.5 mM group showing a slightly higher production, while OCN accumulation in the medium (supernatant) increased significantly as the RGD concentration increases. Serum free medium was used for the last 24 h medium change, hence detected amount of osteocalcin in the medium reflects the accumulation of osteocalcin produced by the cells during the last 24 h. Since osteocalcin is a very small size protein (49 amino acid residues), it can easily diffuse into the medium. Therefore, the amount detected in the medium may be a better indicator of the cell's ability to produce this late bone marker.

Comparable amounts of calcium were detected as RGD concentration increased to 1.25 mM. However, opposed to the increase seen in the ALP and OCN production, a 60% drop was seen in calcium production

as RGD concentration increased from 1.25 mM to 2.5 mM. There are two factors that may be responsible for this decrease in calcium content. First, the three-week timepoint is relatively early for cellular mineralization, as suggested by the high production of early bone marker protein alkaline phosphatase in this experiment. Therefore the detected calcium and mineralization may be largely contributed by nonspecific binding to the hydrogel [20]. Clearing some of the nonspecific binding would indeed facilitate and promote the later cellular mineralization. Therefore, our results suggest that 2.5 mM RGD group provides a better environment for cellular mineralization. Second, mineralization is largely determined by scaffold properties, such as porosity. Incorporating RGD peptide into PEGDA scaffold introduced single ended crosslinking polymer chain into the scaffold, hence decreasing the crosslinking density and increasing the pore size. A larger pore size (i.e. in the 2.5 mM RGD gels) leads to less automineralization, and this may explain the decrease of calcium detected in 2.5 mM RGD group compared to 1.25 mM RGD group.

For the soluble RGD saturation experiment, the cells were incubated in medium containing 0.5 mM soluble RGD both prior to encapsulation and during culture. It has been previously shown that MSC attachment to GRGD-modified hydrogels can be competitively inhibited when cells were incubated in the presence of 0.5 mM soluble GRGD before cell seeding [6]. This indicates that 0.5 mM soluble RGD concentration saturates the cell surface RGD receptors, thus mimicking the highest cell surface RGD concentration scenario. Both quantitative calcium assay and von Kossa staining revealed that the calcium detected in the soluble RGD-treated group was reduced to the same level as the acellular empty gel control, which suggests that only autocalcification contributes to the calcium trapped in the gel and the cells did not produce any calcium. In contrast, the 2.5RGD cellular control without soluble RGD treatment demonstrates calcium production and visible mineralization in both pericellular and intercellular regions. Phosphorous production per cell (by elemental analysis) is also totally blocked by the soluble RGD treatment. All these data imply that maximal RGD concentration would inhibit cellular mineralization process by blocking calcium and phosphate production.

Cbfa-1 was expressed in monolayer culture but the expression was downregulated after being encapsulated into a three-dimensional environment, indicating these cells changed their phenotype when shifted from a two-dimensional to a three-dimensional nonadhesive culture environment. However, the strong expression of cbfa-1 in the 1.25RGD and 2.5RGD group suggests that rendering cells adhesion sites via introducing RGD into the scaffold can help the maintenance of osteogenic potential of MSCs in the three-dimensional PEG hydrogels.

5. Conclusion

In summary, we demonstrated that RGD-conjugated PEGDA hydrogel system promotes the osteogenesis of bone marrow-derived marrow stromal cells. RGD-tethered hydrogel stimulated the production of bone marker proteins, such as alkaline phosphatase and osteocalcin, in a dose-dependent manner, with 2.5 mM being the optimal concentration.

Acknowledgement

We would like to acknowledge NIH, Whitaker Foundation, and Arthritis Foundation for funding.

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