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Osteogenic differentiation of rat bone marrow stromal cells cultured on Arg–Gly–Asp modified hydrogels without dexamethasone and β-glycerol phosphate

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Abstract

In this study, we investigated the effect of signaling peptides incorporated into oligo(poly(ethylene glycol) fumarate) (OPF) hydrogels on in vitro differentiation and mineralization of marrow stromal cells (MSCs) cultured in media without soluble osteogenic supplements (dexamethasone and β-glycerol phosphate). When MSCs were cultured for 16 days on OPF hydrogels modified with Arg–Gly–Asp (RGD) containing peptides, the normalized cell number was dependent on the peptide concentration between days 0 and 5 and reached comparable values at day 10 regardless of the concentration. The alkaline phosphatase (ALP) activity of MSCs on the peptide-modified OPF hydrogels was also concentration-dependent: ALP activity showed peaks on day 10 or day 13 on OPF hydrogels modified with 2.0 and 1.0 μmol peptide/g, which were significantly greater than those on the OPF hydrogels modified with 0.1 μmol peptides/g or no peptide. A characteristic marker of osteoblastic differentiation, osteopontin (OPN), was detected for all the test groups. However, OPN secretion between days 0 and 10 was significantly higher on the peptide modified hydrogels compared to that on tissue culture-treated polystyrene. Taken together, the results indicate that the presence of signaling peptide allows for a favorable microenvironment for MSCs to differentiate into osteoblasts and produce mineralized matrix, although the soluble factors may further enhance calcium deposition. These findings further support the usefulness of OPF hydrogels as scaffolds for guided bone regeneration, and represent an initial step in exploring the complex relationship between soluble and insoluble factors in osteogenic differentiation on biodegradable materials.

Keywords: Osteoblasts; Biomimetic hydrogel; Mineralization; Differentiation; Osteogenic media

1. Introduction

Tissue development is controlled by the interplay of multiple signals that are often provided by extracellular matrix (ECM) proteins [1]. In particular, cells suspended within a 3-dimensional network of ECM respond to external changes via receptor–ligand interactions that relay signals from outside the cell to the cytoskeletal domain and thereby influence subsequent cellular function such as attachment, migration, differentiation, and apoptosis [2]. In order to control and direct specific cellular responses for many tissue engineering applications, significant efforts have been made in the development of new biomaterials to exploit these naturally

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occurring biomolecular signaling processes [3–5]. For example, small cell-binding sequences identified from ECM proteins including Arg–Gly–Asp (RGD), Tyr–Ile–Gly–Ser–Arg (YIGSR) [6], and Arg–Glu–Asp–Val (REDV) [7] have been grafted to the surface of poly(tetrafluoroethylene) (PTFE) [8], metal oxide [9], and glass [10] to enhance the cell attachment and proliferation via receptor-mediated interactions. However, these approaches appear to control cellular response over a relatively short time period (from several hours to a few days in vitro), and modulation of extended tissue-specific function (differentiation of specific cell types) remains a challenge. Furthermore, results from many studies that have been conducted under restricted and well-defined in vitro culture conditions (usually serum-free culture) have not always been useful in predicting the in vivo response of specific tissues to these materials [11].

Recently, we have investigated the potential use of synthetic oligo(poly(ethylene glycol) fumarate) (OPF) hydrogels as biomimetic substrates with chemical and physical features that promote osteoblast function and integration for guided bone regeneration. OPF consists of two major building blocks, poly(ethylene glycol) (PEG) and fumaric acid, which form multiple ester bonds along the linear chain that can be hydrolyzed in a physiological environment leading to degradation of its crosslinked network over time [12]. Importantly, radical polymerization of OPF with water-soluble initiators provides a unique and versatile chemistry in which the concurrent incorporation of cell-binding peptide sequences and in situ hydrogel formation are both possible [13]. In a previous study, we demonstrated that the model peptide sequence, Gly–Arg–Gly–Asp–Ser (GRGDS), was incorporated into the OPF hydrogel, presented through the crosslinked network, and recognized by rat marrow stromal cells (MSCs) [13]. An additional study showed that we could modulate cellular function by altering either the peptide concentration or the nature of the peptide sequence [14]. Specifically, on the OPF hydrogels incorporating a 15 amino acid sequence, Asp–Val–Asp–Val–Pro–Asp–Gly–Arg–Gly–Asp–Ser–Leu–Ala–Tyr–Gly (DVDVPDGRGDSLAYG) derived from osteopontin, MSCs showed faster migration during 3 days of culture than on hydrogels modified with only a GRGDS sequence. In a recent investigation of tissue-specific response to the peptide-modified OPF hydrogel, the signaling peptide presented from the OPF hydrogels directed differentiation of MSCs into osteoblasts in a peptide-concentration-dependent manner, as delineated by expression of osteoblastic phenotypic markers and calcium deposition on the hydrogel [15]. Although it was evident in these studies that the peptide-modified OPF hydrogel serves as a temporary substrate for MSCs to undergo in vitro differentiation and mineralization, the potential contribution of culture media to this specific cellular response was not clearly addressed. We have cultured MSCs in Dulbecco's modified Eagle's medium (DMEM) with osteogenic supplements including dexamethasone, β-glycerol phosphate, and L-ascorbic acid. In this system, decoupling the contributions from supplements in the media vs. the peptide in the hydrogel toward in vitro differentiation and mineralization of MSCs was difficult. In particular, dexamethasone has been reported to be important in the differentiation of osteoprogenitor cells isolated from young adult rat bone marrow stroma [16,17]. These concerns led us to conduct an additional experiment to assess the effects of soluble signaling factors in the culture media on differentiation and mineralization of MSCs cultured on a peptide-modified hydrogel.

In this study, we tested the hypothesis that signaling peptides incorporated into the OPF hydrogels induced differentiation and mineralization of MSCs cultured in the absence of dexamethasone and β-glycerol phosphate. In order to address this hypothesis we examined change in the cell number, expression of typical in vitro osteogenic differentiation markers (alkaline phosphotase (ALP) activity and osteopontin), and calcium deposition from MSCs cultured for 16 days on OPF hydrogels modified with two different peptides.

2. Materials and methods

2.1. Materials

PEG-diacrylate (PEG-DA) (molecular weight (MW) 575), PEG (nominal MW 1000), ammonium persulfate (APS), and triethylamine were purchased from Aldrich (Pittsburgh, PA). Fumaryl chloride was purchased from Acros (Milwaukee, WI) and distilled prior to use. Ascorbic acid (AA) was obtained from Sigma (Saint Louis, MO). Acryloyl-PEG-N-hydroxysuccinimide (acryloyl-PEG-NHS) (MW 3 400) was purchased from Shearwater Polymers (Huntsville, AL). DMEM and trypsin/EDTA were purchased from Gibco Life (Grand Island, NY) and fetal bovine serum (FBS) was obtained from Gemini Bio-Products (Calabasas, CA). Phosphate buffered saline (PBS) and antibiotic-antimycotic solution (penicillin/streptomycin) were purchased from Invitrogen (Carlsbad, CA). A synthetic cell binding peptide derived from fibronectin, GRGDS, was purchased from Bachem California (Torrance, CA). An ABI 433A Peptide Synthesizer (PE Biosystems, Foster City, CA) was used at the Mayo Protein Core Facility (Rochester, MN) to synthesize a 15 amino acid OPN-derived peptide (ODP), DVDVPDGRGDSLAYG. The resulting product has its composition further verified by electrospray ionization (ESI) mass analysis on a Perkin/Elmer Sciex API 165 Mass Spectrometer (PE Biosys-

tems, Foster City, CA). Unless otherwise stated, the reagents were used as received.

2.2. Synthesis of oligo(PEG fumarate) (OPF) and acrylation of peptides

OPF was synthesized following a previously established procedure [12]. Briefly, PEG was dissolved in toluene and dehydrated by azeotropic distillation. Triethylamine and distilled fumaryl chloride were dropped concurrently into the PEG solution in anhydrous tetrahydrofuran and mixed in an ice bath overnight. Following solvent evaporation, the resulting product was recrystallized in ethyl acetate and precipitated in anhydrous ethyl ether. The remaining solvents were removed at 0.1 Torr for at least 5 h. The molecular weight of OPF was determined by gel-permeation chromatography (GPC) using mono-dispersed PEG standards. The dried OPF was stored in vacuum at 0°C prior to use.

The synthetic peptides GRGDS and ODP were acrylated as described previously [18]. Briefly, the peptides were dissolved in sodium bicarbonate buffer solution (pH 8.3) and acryloyl-PEG-NHS was reacted with the peptide at room temperature for 2.5 h. The reaction mixture was dialyzed in deionized distilled water (DDW) for 2 days using a dialysis membrane (MW cutoff: 2 000) to remove any unreacted residues. The dialyzed polymer solution was lyophilized overnight and stored at 0 °C prior to use.

2.3. Preparation of peptide-modified OPF hydrogels

OPF hydrogels were chemically crosslinked with PEG-DA in the presence of APS and AA as initiators in the following manner: OPF and PEG-DA were dissolved in DDW at 50% (w/v). The amount of added PEG-DA to OPF was determined by the ratio of the number of double bonds in PEG-DA to those in OPF (DBR). Specifically, 0.123 mL PEG-DA was mixed with 0.2 g OPF in 0.667 mL DDW for preparation of the OPF hydrogel with a DBR equal to 3. The number of double bonds in OPF was calculated based on the number average molecular weight of the synthesized OPF as previously described [13]. Various amounts of peptide (0.1, 1.0, or 2.0 μmol acrylated peptide/g of the swollen hydrogel) was then added to the aqueous mixture of OPF and PEG-DA with APS and AA in an equal concentration of 0.025 M for the crosslinking reaction (the weight of the swollen hydrogel was previously reported [13]). Subsequently, the mixture was cast between two glass plates and allowed to crosslink at 45°C for 30 min. After crosslinking, the hydrogel film was lifted from the plate using a razor blade and then immersed in DDW for 24 h in order to remove any remaining residues. The fully swollen hydrogel film was cut using a cork borer 15 mm in diameter. We analyzed release of the peptide from the hydrogel during swelling by using NMR and no free peptide was detected (data not shown). For sterilization, the hydrogel film was soaked in 70% ethanol (v/v) for 1 day and re-swollen in sterile phosphate buffered saline (PBS) solution for 2 days while changing the PBS twice per day under sterile conditions. Two types of the OPF hydrogels were prepared as shown in Table 1: OP-RGD incorporating the GRGDS sequence and OP-ODP incorporating the ODP.

2.4. Isolation of marrow stromal cells (MSCs)

We isolated MSCs from male Wistar rats (Harlan, Indianapolis, IN) (140–160 g) as previously described [13]. Briefly, femurs and tibias were excised under aseptic conditions and the marrow was flushed using a 20G needle with DMEM supplemented with 5% (v/v) antibiotics containing penicillin and streptomycin (pH 7.2). The flushed medium was centrifuged and the supernatant was discarded. The remaining cell pellet was suspended in a volume of 3 mL culture media (DMEM supplemented with 10% (v/v) FBS, 1% (v/v) antibiotics containing penicillin and streptomycin, and 50 mg/L L-ascorbic acid) and seeded on T-75 culture flasks. After 3 days, non-adherent cells were aspirated and the cell culture was replenished with fresh culture media.

Table 1
Name and chemical compositions of prepared OPF hydrogels

Name of OPF[a] hydrogel film	DBR[b]	Types of incorporated peptide	Concentrations (μmol/g)
OP-ODP	3	ODP[c] (DVDVPDGRGDSLAYG)	2.0, 1.0, or 0.1
OP-RGD	3	GRGDS	2.0, 1.0, or 0.1

[a]The number average molecular weight (M_n) of PEG and its corresponding OPF were 930 ± 10 and 4470 ± 60, respectively. M_n was determined using gel permeation chromatography relative to a calibration curve with mono-dispersed PEG standards.

[b]Ratio of double bonds present in PEG-DA to those in OPF. Weight ratio in aqueous mixture of PEG-DA and OPF was determined based on double bond ratio (DBR).

[c]ODP: osteopontin-derived peptide.

2.5. MSC culture on the peptide-modified OPF hydrogels

The sterilized circular peptide-modified hydrogel films were placed in 24-well tissue culture plates (flat bottom with lid, Corning, NY). Cylindrical stainless steel annuli (7.2 mm in inner diameter, 15.8 mm in outer diameter, and 16 mm in height) were autoclaved and placed on the top of the hydrogel films to prevent movement of the hydrogel films. On day 6 of culture following the isolation, the MSCs were lifted using 0.05% trypsin/0.53 mM EDTA solution and re-suspended with the culture media at a concentration of 132,750 cells/mL. A cell suspension of 170 μL was seeded onto the confined area of each hydrogel film (39,750 cells/cm^2) inside the stainless-steel annuli. The cell suspension at the same concentration was seeded on tissue culture polystyrene as a control. Cell cultures were maintained under standard culture conditions (37°C, 95% relative humidity, and 5% CO_2) for periods as long as 16 days with media changes every 2–3 days. During the culture, the morphology was monitored via light microscopy. At the given time points, the samples were removed from culture and subjected to biochemical assays.

2.6. Sample preparation for assays

Samples were prepared for measuring cellularity, ALP activity, and calcium content in the following manner. On days 5, 10, 13, and 16, the cultured layer of MSCs on the peptide-modified hydrogel was rinsed with PBS twice and scraped carefully using a plastic cell scraper. 1 mL of lysis buffer (25 mM Tris and 0.5% (v/v) Triton X-100 (pH~7.4) [19]) was then added to the scraped cell pellet and the cell lysates were frozen immediately and stored at −80°C until the day of the assays when the cell lysates were subjected to three thaw/freeze cycles (20 min with sonication at 37 °C and freezing at −80 °C for 20 min) three times for complete extraction of DNA and proteins from the cell cytoplasm. For analysis of OPN secretion, 200 μL of the cell culture media was collected from each group every 2 or 3 days and stored at −80 °C.

2.7. Analysis of cellularity

The number of cells on the peptide-modified hydrogel at each time point during the cell culture was measured using a fluorometric PicoGreen DNA kit (Molecular Probes, Eugene, OR) that quantifies the amount of double stranded DNA in cells. The fluorescent absorbance of samples was measured using a microplate fluorescent reader (FL × 800, BIO-TEK Instrument, Winooski, VT) equipped with a 480/525 (excitation/emission) filter set. A standard curve based on known concentrations of calf thymus DNA was used to determine the total cell number. The cell number was normalized at each time point to the number of initially seeded cells.

2.8. ALP activity assay

ALP activity was measured according to a previously published method using Sigma Diagnostic Kit #104 [20]. Aliquots of the lysates (80 μL), 20 μL of alkaline working buffer solution (5 mM $MgCl_2$ and 0.5 M 2-amino-2-methyl-1-1-propanol), and 100 μL of substrate solution (5 mM *p*-nitrophenylphosphate) were added to a 96-well plate and incubated for 1 h at 37°C. Standards in known concentrations ranging from 50 to 250 μM were prepared from serial dilutions of the stock solution provided from the kit and added to designated wells in the same plate containing the samples. After incubation, the kinase reaction was stopped by adding 100 μL of 0.3 M NaOH to each well. The absorbance of each well was measured using a microplate reader (PowerWave 300, BIO-TEK Instrument, Winooski, VT) at 405 nm. The results were normalized to the total cell number at each time point.

2.9. Osteopontin (OPN) assay

The OPN secretion was analyzed using a rat OPN TiterZyme enzyme-linked immunosorbent assay (ELISA) Kit (Assay Designs, Inc., MI) [21]. Briefly, the samples from the media were collected and grouped, representing the time periods of days 0–5, 5–10, 10–13, and 13–16. The results represent the OPN secretion per day over each time period. For the assay, a volume of 100 μL assay buffer provided from the kit and 100 μL of the samples or standard solution were added into a microtiter plate. A series of standard solutions were prepared by diluting the stock solution containing recombinant rat OPN (9.5 μg/mL). The microtiter plate with the samples was incubated at 37 °C for 1 h, the solutions in the well were aspirated, and the well was rinsed by adding 300 μL of a washing buffer six times. Then, 100 μL of the labeled antibody solution (in PBS) was added to each well and the plate was sealed and incubated at 4°C for 30 min. The antibody solution was aspirated and the well was rinsed using 300 μL of the washing buffer eight times. Subsequently, a volume of 100 μL of a substrate solution was added to each well and incubated for 30 min at room temperature in the dark. The reaction was stopped by the addition of 100 μL of a 1N sulfuric acid solution (in DDW) to each well. The absorbance of each well at 450 nm was measured using the microplate reader.

2.10. Analysis of calcium content

The calcium content in the cell lysates were measured according to a previously published method using Sigma

Diagnostic Kit # 587-A [20], which measures the amount of purple-colored calcium-OCPC (*o*-cresolphthalein complexone) complex formed as a result of binding between OCPC and free calcium generated by osteoblasts. A volume of 0.5 mL lysates was incubated at room temperature overnight with 0.5 mL acetic acid (1.0 N) in order to extract calcium from mineralized constructs. A volume of 10 μL from the samples was taken and placed in a 96-well plate. Equal amounts (150 μL) of calcium binding reagent (*o*-cresolphthalein complexone, 0.24% 8-hydroxyquinoline, and surfactant) and calcium buffer reagent (500 mM 2-amino-2-methyl-1,3-propanediol) provided from the kit were added to each well and the plate was incubated at room temperature for 10 min. The standards in concentrations ranging from 5 μg/mL to 100 μm/mL were prepared using $CaCl_2$. The absorbance of each well was measured using a microplate reader at 575 nm. The calcium content from each sample was normalized to the total cell number at each time point.

2.11. Statistical analysis

The results were reported as means ± standard deviation of each experiment in triplicate. Multivariate analysis of variance (MANOVA) was used to determine if there was any significant difference between any of the data sets utilizing SYSTAT 8.0 software packages for Windows. Tukey multiple comparison tests were subsequently performed to determine which pairs were significantly different. Both tests were conducted with a 95% confidence interval ($\alpha = 0.05$). Because plateau values were reached for the total cell number after 5–10 days, no logarithmic transformation was needed before the statistical analysis was completed for the data.

3. Results

3.1. Analysis of cellularity

The number of MSCs on OP-ODP increased with increasing peptide concentration between days 0 and 5, where the normalized cell number was 1.6 ± 0.3, 1.2 ± 0.3, and 0.7 ± 0.2 on OP-ODP modified with 2.0, 1.0, and 0.1 μmol peptide/g, respectively. However, Fig. 1 shows that the normalized cell number reached a plateau value at day 10 and remained constant during the rest of the cell culture period. Similarly, at day 5, the normalized cell number of MSCs on OP-RGD modified with 2.0 μmol peptide/g (2.1 ± 0.1) was significantly higher than that on OP-RGD modified with both 1.0 μmol peptide/g (1.3 ± 0.0) and 0.1 μmol peptide/g (0.4 ± 0.1). The normalized cell number for OP-RGD modified with 2.0 μmol peptide/g did not change from the initial value of day 5 over 16 days of the culture

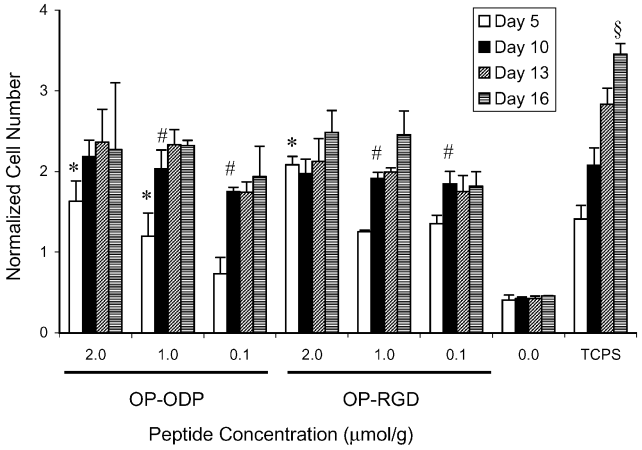

Fig. 1. Proliferation of MSCs cultured on OP-ODP and OP-RGD of different peptide concentrations, the unmodified hydrogel, and tissue culture polystyrene (TCPS) controls as a function of the culture time. The *Y*-axis represents the normalized cell number obtained by dividing the cell number at given time points with the number of initially seeded cells. $^{*}p < 0.05$ relative to OP-ODP or OP-RGD hydrogels modified with 0.1 μmol/g at day 5. $\#p < 0.05$ relative to day 5 for each group. $\S p < 0.05$ relative to days 5, 10, and 13. Error bars represent means ± standard deviation for $n = 3$.

period, while the normalized cell numbers at day 16 for OP-RGD modified with both 1.0 μmol peptide/g (2.5 ± 0.3) and 0.1 μmol peptide/g (1.8 ± 0.2) were significantly increased compared to those at day 5. These data correlate to what was observed over the culture period via light microscopy. Cells maintained an elongated morphology until they reached confluency at 3–5 days after seeding, depending on the peptide density. After this point, visualization of individual cell morphology became increasingly difficult. Approximately 40% of the initially seeded cells adhered to the OPF hydrogel without the peptides, and this cell number remained constant over the 16-day culture period. The normalized cell numbers on OP-RGD and OP-ODP modified with 2.0 and 1.0 μmol peptide/g were comparable to those on TCPS through day 13, but were significantly lower than that of TCPS at days 13 and 16.

3.2. ALP activity

ALP activity of MSCs on the peptide-modified hydrogel was dependent on the peptide concentration (Fig. 2); the general trends of ALP activity over the 16 days of culture were as follows: (1) a continuous increase in ALP activity for the hydrogel modified with 2.0 μmol peptide/g, (2) an increase in ALP activity between days 0 to 13 and a decrease to day 16 for the hydrogel modified with 1.0 μmol peptide/g, and (3) limited ALP activity during the entire culture period for the hydrogels modified with 0.1 μmol peptide/g and no peptide. At day 5, ALP activity of MSCs on OP-ODP modified with

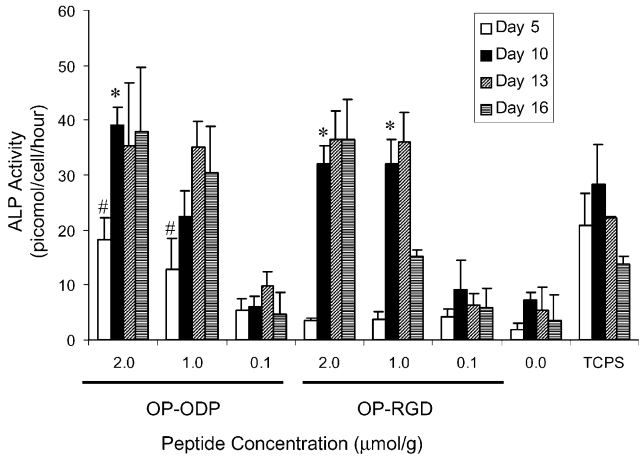

Fig. 2. ALP activity of MSCs on OP-ODP and OP-RGD of different peptide concentrations, the unmodified hydrogel, and TCPS controls as a function of the culture time. ALP was measured using an end point enzyme activity assay and the results are expressed as picomol/cell/h. $^{*}p<0.05$ relative to day 5 for each group. $\#p<0.05$ relative to OP-RGD modified with the same peptide concentration as respective OP-ODP. Error bars represent means $\pm$ standard deviation for $n=3$.

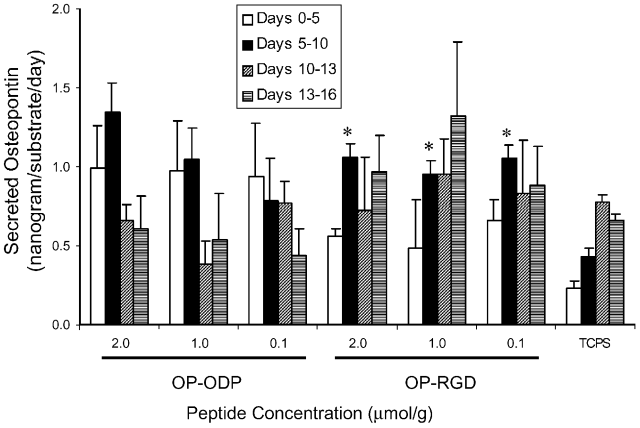

Fig. 3. Amount of secreted OPN from cultured MSCs on OP-ODP and OP-RGD of different peptide concentrations and TCPS controls during the culture periods designated by the time intervals on the graph. Results are expressed as nanogram/substrate/day. $^{*}p<0.05$ relative to day 5 for each group. Error bars represent means $\pm$ standard deviation for $n=3$.

2.0 and 1.0 μmol peptide/g was 18.3 ± 3.9 and 12.8 ± 5.6 pmol/cell/h, respectively, which was greater than those on OP-RGD hydrogels modified with the same concentration of the peptide (3.6 ± 0.4 and 4.3 ± 1.4 pmol/cell/h, respectively). On TCPS controls, ALP activity showed the maximum (28.3 ± 7.3 pmol/cell/h) at day 10 and gradually decreased during the rest of the 16-day cell culture period.

3.3. OPN secretion

Fig. 3 shows quantitative analysis of OPN secretion from MSCs cultured on the peptide-modified hydrogels over 16 days. To obtain these data, the media was collected every 2 or 3 days during regular media changes, the total amount of OPN in the accumulated media was quantified using ELISA, and results are presented as the OPN secretion per day. On OP-ODP modified with 2.0 and 1.0 μmol peptide/g, the amount of OPN secretion exhibited an increase between days 0 to 10 followed by a decrease over the rest of the culture period. For example, the amount of OPN secretion on OP-ODP modified with 2.0 μmol peptide/g between days 5 and 10 was 1.3 ± 0.2 ng/substrate/day and decreased to 0.6 ± 0.2 ng/substrate/day between days 13 and 16. On OP-RGD, the general trend was evident that the amount of OPN secretion between days 5 and 10 was significantly greater than those between days 0–5, and remained almost constant thereafter. Between days 0 and 10, OPN secretion on TCPS was significantly lower than that on peptide-modified hydrogels. On the TCPS control, the amount of OPN in the media showed a typical rise–drop pattern and reached the maximum between days 10 and 13.

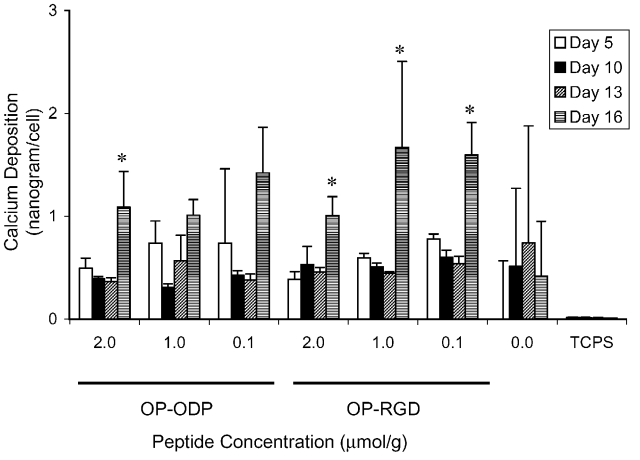

Fig. 4. Amount of deposited calcium from the culture of MSCs on OP-ODP and OP-RGD of different peptide concentrations, unmodified hydrogels, and tissue culture polystyrene (TCPS) controls as a function of the culture time. The total calcium content at each time point was normalized to the cell number for each experimental group. $^{*}p<0.05$ relative to day 5 for each group. Error bars represent means $\pm$ standard deviation for $n=3$.

3.4. Analysis of calcium content

The analysis of calcium deposition from MSCs on the peptide-modified hydrogels is shown in Fig. 4. Overall, there is increase of calcium deposition on the peptide-modified hydrogels over the 16-day culture period. On OP-RGD, the calcium content at day 16 was 1.0 ± 0.2, 1.7 ± 0.8, and 1.6 ± 0.3 ng/cell for peptide concentrations of 2.0, 1.0, and 0.1 μmol peptide/g, respectively, and all

of these were greater than their corresponding values at day 5. It is notable that no calcium was detected from MSCs cultured on TCPS controls under these culture conditions during the entire culture period.

4. Discussion

The objective of this study was to test the hypothesis that ECM protein-derived cell-adhesive peptide sequences incorporated into OPF hydrogels induce in vitro differentiation and mineralization of MSCs cultured in the absence of the osteogenic supplements dexamethasone and β-glycerol phosphate. Dexamethasone is a synthetic glucocorticoid shown to induce osteogenic differentiation in both rat and human MSCs [22–26], and β-glycerol phosphate [23] promotes mineralized matrix formation by acting as a potential source of phosphate ions. For clarification, herein, the media without osteogenic supplements are designated "no-DEX/βGP" as compared to "with-DEX/βGP" media, which contains these supplements.

A previous study in our laboratory demonstrated that MSCs on peptide-modified hydrogels expressed markers of osteoblast differentiation and deposited significantly higher amounts of calcium compared to TCPS controls during 16 days of culture in the with-DEX/βGP media. More importantly, the differentiation and mineralization of MSCs on the peptide-modified hydrogels were enhanced with increasing peptide concentrations. Because other work suggested that there was little hydrogel degradation over the first 16 days [27], it is believed that the cells in this case were interacting primarily with the peptide as it was bound to the hydrogel. However, it was unclear whether the enhanced differentiation and mineralization of MSCs in this experiment was due to the combined effect of the osteogenic supplements in the media and cell attachment to the peptides on the hydrogels, or was mainly due to one or the other of these two factors. Such questions led us to conduct this study where we investigated the in vitro differentiation and mineral deposition from MSCs in the no-DEX/βGP media. However, when comparing the results between the previous and current studies, it is important to note that different cell isolation were used for the two experiments. The source of the cells has been previously shown to influence overall calcium deposition from MSCs encapsulated within OPF hydrogels cultured in otherwise identical conditions [28].

The overall normalized cell number showed a 2–3-fold increase relative to the number of initially seeded cells during the 16 days of culture. The MSC cell number was dependent on the peptide concentration in that peak levels were achieved at earlier time points with the higher peptide concentrations; the peak was at day 5 for 2.0 μmol peptide/g modified hydrogels while it occurred at day 10 for the lower peptide concentration hydrogels. The normalized cell number on TCPS was significantly higher than that for the hydrogels at day 16. The overall normalized cell number on the peptide-modified hydrogels cultured in the no-DEX/βGP media was consistent with our previous results in the with-DEX/βGP media, indicating that the supplements in the media appear unlikely to affect the attachment and/or proliferation of MSCs [15].

In the previous study, the with-DEX/βGP media group demonstrated that the ALP activity of MSCs cultured on the peptide-modified hydrogels exhibited a typical rise–fall pattern graph for all culture conditions [15], as has been reported elsewhere for differentiating osteoblasts in vitro [21]. However, the ALP activity of cultured MSCs in the no-DEX/βGP media showed similar trends only for the hydrogels modified with 2.0 and 1.0 μmol peptide/g. ALP activity of MSCs cultured on the hydrogel at 0.1 μmol peptide/g was significantly decreased to basal level, which was defined to be the amount produced by cells on the hydrogel without the peptides. Notably, the maximal ALP activity of MSCs on hydrogels modified with 2.0 and 1.0 μmol peptide/g in the no-DEX/βGP media maintained almost the same level as that in the with-DEX/βGP media presented in the previous study [15]. These findings are somewhat unanticipated given that previous reports demonstrated that the number of ALP-positive MSC colonies in dexamethasone-free media was significantly lower than that in the media with dexamethasone [22]. These data clearly suggest that the signaling peptide presented by the OPF hydrogels provided sufficient biomolecular cues to promote early differentiation of MSCs over TCPS, independent of the presence of osteogenic supplements in the media. Additionally, the significant decrease in ALP activity of MSCs on the hydrogels modified with 0.1 μmol peptide/g further supports the idea that the presence of the peptide is an important signal for in vitro differentiation of MSCs under the no-DEX/βGP condition. Thus, the presence of soluble differentiation factors may be required to optimize early cell differentiation toward the osteoblastic phenotype only at lower peptide concentrations.

OPN is a 60 kDa acidic phosphoprotein that possesses several calcium binding domains and is associated with cell attachment, proliferation, and mineralization of ECM into bone [29]. Importantly, OPN, at the mRNA and protein levels, is actively expressed in the proliferation stage of osteoblasts as an early marker of osteoblast differentiation, decreases postproliferatively, and then increases at the onset of matrix mineralization [30]. A study with titanium fiber mesh scaffolds seeded with MSCs cultured under flow conditions revealed the temporal pattern of OPN secretion in the culture media with peak levels occurring between days 3–5 and days 8–11 depending on the culture conditions [21]. Although

direct comparison of these results with ours is not possible due to differences in the two culture systems, our results demonstrating increasing secretion of OPN between days 0–10 indicate the early differentiation of MSCs into osteoblasts on the peptide-modified hydrogels in the no-DEX/βGP media. Interestingly, the delay of the peak secretion of OPN on TCPS to days 10–13 suggests an acceleration of the osteoblast differentiation on the peptide-modified hydrogels. The temporal secretion of OPN on OP-ODP is similar to that previously observed on the same hydrogel in the with-DEX/βGP media with respect to the culture time needed to reach the peak as well as the peak value of the secreted OPN. Therefore, we speculate that the signal presented from the peptide incorporated in the OPF hydrogel may be sufficient to induce the peak of OPN secretion at a saturated level or that soluble factors may not be necessary for secretion of this specific protein.

The overall amount of calcium deposited on the peptide-modified hydrogel was limited in the no-DEX/βGP media, although there was a trend of increasing calcium content on OP-RGD hydrogels over 16 days of culture. Calcium deposition is a late marker of osteogenic differentiation [21]. While direct comparison with the previous study (with DEX/βGP media) is not possible due to the lack of β-glycerol phosphate in the media in this study, which could decrease mineralization due to a reduction in phosphate ion concentration, these findings demonstrate the continued osteoblastic differentiation and matrix production of MSCs cultured on the peptide-modified hydrogels. The importance of signaling peptides in enhanced mineralization has been addressed in recent studies using mature osteoblasts. Rezina and Healy demonstrated an effect of peptide density on long-term matrix production on a bone sialoprotein-derived peptide modified surface [31]. In addition, osteoblasts exhibited enhanced mineralization when encapsulated in PEG-based hydrogels tethered with RGD [32]. Taken together, our data indicate that the presence of signaling peptides allows for a favorable microenvironment for osteoprogenitors in MSCs to differentiate into osteoblasts and produce calcified ECM, although the soluble factors dexamethasone and β-glycerol phosphate may further enhance calcium deposition.

In the previous study, we demonstrated that the levels of the phenotypic markers associated with osteoblast differentiation and mineralization were not significantly affected by the nature of the peptide sequence [15]. We included the same two peptide sequences in these experiments to examine the effects of peptide type in the no-DEX/βGP media. As observed in the previous study, the differentiation and mineralization of the MSCs was not dependent on the peptide sequences used, suggesting that the RGD peptide sequence, as a primary cell binding domain in ODP, plays a main role in MSC attachment, and further differentiation of MSCs into osteoblasts. Since it has been established that RGD interacts with a variety of cell integrin receptors [33–36], this finding further indicates that this receptor class may play a role in MSC differentiation, even in the absence of stimulatory factors such as dexamethasone. It is unclear from these experiments, however, if peptide binding increases the proliferation and differentiation of the subpopulation of DEX-independent cells found previously to exist within rat MSC isolation [22], if the peptide–receptor interactions act as a "surrogate" for dexamethasone in promoting differentiation of the DEX-dependent subpopulation, or if a combination of these events occurs.

The strategy proposed in this project envisions the recruitment and attachment of pluripotent, mesenchymal cells onto the biodegradable and biomimetic hydrogels, and their subsequent commitment toward the osteoblast phenotype in order to aid regeneration of bone. Interaction of the biomimetic hydrogel with surrounding cells is crucial to allow bone cell growth, differentiation, and mineralization. Since bone development is a complex process, it appears to be difficult to modulate each sequential stage of cellular function solely by a single cell-binding peptide sequence. A number of molecules including growth factors, cytokines, and other chemical modulators have been identified to have differential effects on cells involved in bone development. Therefore, OPF hydrogels that incorporate a combination of multiple signaling domains may be the next step toward designing ideal materials for controlling multiple responses of bone forming cells. Additionally, as demonstrated by numerous studies using inorganic materials with improved osteoblast mineralization due to structural similarity to the mineral phase of bone [37–39], introduction of these inorganic components such as hydroxyapatite and calcium phosphate in the OPF hydrogel to form a composite material may enhance mineralization of osteoblasts. Since the OPF hydrogels are extremely versatile, further in situ modification with multiple peptides and/or proteins could be efficiently achieved based on a similar water-soluble initiation scheme as that employed in these studies.

5. Conclusions

Biomimetic OPF hydrogels were prepared using two types of peptide sequences with varied peptide concentrations in the hydrogel. MSCs derived from young adult rat bone marrow were then cultured on these hydrogels for 16 days in media lacking the osteogenic supplements dexamethasone and β-glycerol phosphate and their differentiation and mineralization potential were examined. We observed a significantly higher cell

number on peptide-modified OPF hydrogels than non-modified hydrogels in the no-DEX/βGP culture, regardless of the peptide type. MSCs in the no-DEX/βGP media showed typical trends of ALP activity, with a transient up- and down-regulation over the 16-day culture period. Another characteristic marker of osteogenic differentiation, OPN, was detected for all experimental groups in the study and was significantly increased for the peptide-modified hydrogels over TCPS controls. Despite evident osteoblastic development of MSCs on the peptide-modified hydrogel, calcium deposition was limited during the 16 days of culture. Taken together, the results indicate that the presence of signaling peptides allows for a favorable microenvironment for MSCs to differentiate into osteoblasts and produce mineralized matrix and further support the usefulness of OPF hydrogels as scaffolds for guided bone regeneration.

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