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Modular Peptides Promote Human Mesenchymal Stem Cell Differentiation on Biomaterial Surfaces

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Summary

Molecular design strategies in biomedical applications often involve creating modular “fusion” proteins, in which distinct domains within a single molecule can perform multiple functions. We have synthesized a new class of modular peptides that include a biologically active sequence derived from the growth factor BMP-2 and a series of hydroxyapatite-binding sequences inspired by the N-terminal α -helix of osteocalcin. These modular peptides can bind in a sequence-dependent manner to the surface of “bone-like” hydroxyapatite coatings, which are nucleated and grown on a biodegradable polymer surface via a biomimetic process. The BMP2-derived sequence of the modular peptides is biologically active, as measured by its ability to promote osteogenic differentiation of human mesenchymal stem cells. Our study indicates that the modular peptides described here are multifunctional, and the characteristics of this approach suggest that it can potentially be applied to a range of biomaterials for regenerative medicine applications.

Keywords

Bone regeneration; bone morphogenetic protein; osteocalcin; hydroxyapatite; osteogenesis

1. Introduction

Natural proteins often contain at least two functional domains, which are linked together to form one multi-functional protein molecule. Extracellular matrix (ECM)-binding cell adhesion proteins in mineralized tissues provide illustrative examples of this “modular” protein design. For example, the bone ECM protein osteocalcin (OCN) binds to the hydroxyapatite mineral component of bone with high affinity via an N-terminal domain [1,2], and also plays a critical role in regulating bone matrix formation via a C-terminal domain [1]. Stayton and coworkers

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recently demonstrated that it is possible to mimic the modular properties of natural proteins by engineering a synthetic peptide that binds to hydroxyapatite (HA) via a statherin-derived unit and also remains capable of affecting cell adhesion via an Arg-Gly-Asp (RGD) cell adhesion unit [3]. Specifically, these peptides showed a strong interaction with HA through N-terminal acidic sequence of statherin and promoted $\alpha_v\beta_3$ integrin-mediated adhesion of a Mo α_v melanoma cell line. These natural and synthetic examples demonstrate that modular peptide design can be used as a strategy to promote cell adhesion to a common natural and biomedical material - hydroxyapatite. In the current study we hypothesized that the concept of modular peptide design could be extended in two important ways. First, we hypothesized that modular peptides could include a growth factor-derived component to induce stem cell differentiation. Second, we focused on binding of modular peptides to “bone-like” HA coatings grown on standard biodegradable polymers, in order to explore the applicability of this approach to a common class of biodegradable materials.

The approach described in this manuscript was designed to promote differentiation of human mesenchymal stem cells (hMSCs) into osteoblasts. MSCs are capable of differentiating into multiple cell lineages, including osteoblasts, chondrocytes, and adipocytes [4–6]. Osteoblast differentiation has been shown to be regulated by multiple proteins, including bone morphogenetic proteins (BMPs) and Wnt [7–9]. Among them, BMP-2 is one of the most potent inducers of osteogenic MSC differentiation *in vitro* and *in vivo* [8]. BMP-2 promotes osteogenic differentiation by up-regulating expression of bone-related proteins, including osteocalcin (OCN), osteopontin (OPN), and alkaline phosphatase (ALP) [7,10]. Previous studies have demonstrated that various forms of BMP-2 are capable of inducing bone formation at ectopic and orthopic sites, including recombinant human BMP-2 protein [11,12]. Recently a 20-mer synthetic peptide (KIPKASSVPTELSAISTLYL) derived from the knuckle epitope of BMP-2 protein was developed and found to induce osteogenic differentiation of the multi-potent C3H10T1/2 cell line [13], ectopic bone formation [14], and orthotopic bone formation [15]. Taken together, these studies indicate that multiple forms of BMP-2 are capable of inducing differentiation of precursor cells down the osteoblast lineage.

Based on the multifunctional properties of natural skeletal proteins (e.g. osteocalcin) and the inductive effects of BMP-2 on hMSC differentiation, we have developed a modular peptide design strategy that combines a HA mineral-binding unit and a BMP-2-derived unit. The HA-binding unit is inspired by the 5.7kDa natural protein osteocalcin (OCN) [16]. Osteocalcin-HA binding is largely mediated via a peptide sequence (γ EPRR γ EV γ EL: 17–25), which contains three unusual γ -carboxylated glutamic acid (γ E; Gla) residues at positions 17, 21 and 24. These Gla residues coordinate with calcium ions in the HA crystal lattice to promote high levels of binding [17]. The BMP2-derived unit is the 20-mer peptide previously shown by Tanihara and coworkers to display the biological activity of full length BMP-2 protein [13].

Our results demonstrate that these modular peptides are capable of binding to the surface of a “bone-like” HA coating, which is formed on a poly(lactide-co-glycolide) surface via a biomimetic process used previously by us and several other groups [18]. The binding efficiency and subsequent release of the modular peptides from HA coatings can be adjusted by rationally varying the OCN-inspired sequences. In addition, the BMP-2-derived portion of these molecules is biologically active, as it is capable of promoting osteogenic differentiation of hMSCs on the surface of HA coatings *in vitro*.

2. Materials and Methods

2.1. Synthesis and purification of modular growth factors

Multiple modular peptides (Table 1) were synthesized by solid-phase peptide synthesis on Fmoc-Rink Amide MBHA resin with Fmoc-protected α -amino groups via peptide synthesizer

(CS Bio, Menlo Park, CA) or manually with PyBop/DIPEA/HOBt activation. The side-chain-protecting groups used were: t-butyl for Tyr, Thr and Ser; 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl for Arg; t-BOC for Lys; and t-butyl ester for Glu and Asp. In some cases, 5(6) - FAM (5(6) - carboxyfluorescein, Sigma) was conjugated to the N-terminal lysine residue to characterize binding and release kinetics of modular growth factors on HA-coated substrates (HPS). The resulting peptide molecules were cleaved from resin for 4 hr using a TFA:TIS:water (95:2.5:2.5) cocktail solution, filtered to remove resin, and precipitated in diethyl ether. Crude peptide mixtures were purified using a Shimadzu Analytical Reverse Phase-HPLC (Vydac C18 column) with 1%/min of 0.1% TFA in acetonitrile (ACN) for 60 min and analyzed by MALDI-TOF mass spectrometry (Bruker Reflex II time-of-flight mass spectrometer).

2.2. PLG film preparation and mineral growth

PLG films were prepared via a solvent casting process in which poly (lactide-co-glycolide) (85:15) pellets were dissolved in chloroform (50 mg/ml), added to a PTFE dish, and dried for 2 days. The films were further dried at 50–55 °C for 4 hr to remove residual solvent and samples were cooled to RT. Square films (1 cm²) were manually cut out of the resulting PLG sheets. A bone-like HA surface was grown on PLG films using a previously described approach [19].

The surface morphologies of HA-coated and uncoated PLG films were examined by scanning electron microscopy (SEM). A conductive gold coating was applied to the surface of each film via sputter coating, and samples were imaged under high vacuum using a LEO 1530 SEM (Zeiss, Oberkochen, Germany) operating at 10–30 kV. X-ray diffraction spectra of mineral-coated HPS and non-coated PLG films were collected using a Bruker Hi-Star 2-D X-ray diffractometer (XRD).

2.3. Binding isotherms and release kinetics of modular peptides

To measure the binding efficiency of modular peptides to the HA-coated PLG substrates (HPS) and to gain preliminary insight into the properties that influence modular peptide immobilization, we first exposed 1 cm² HPS substrates to PBS solutions containing 500 µg (1 mg/ml) of 5(6) FAM-conjugated eBMP-2, eBGU1, eBGU3, eBGAL1, or eBGAL3 modular peptide solution (See Table 1 for definitions of these abbreviations). HPS were incubated in peptide solutions with constant agitation for 4 hr at 37 °C, and the amount of free peptide remaining was determined by measuring the fluorescence emission of the solution (excitation: 494 nm; emission 519 nm) using a Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT), and comparing this emission to standard samples with known concentrations of 5(6)-FAM. To further characterize surface immobilization of the peptide with the highest binding efficiency - the eBGAL3 peptide - 1 cm² HPS were incubated in various concentrations (50–750 µM) of 5(6)-FAM-conjugated eBGAL3 peptide for 4 h with constant agitation at 37 °C. The amount of peptide bound to HPS was again determined by fluorescence analysis, as described above. To quantify release kinetics of 5(6) FAM-conjugated modular peptides from HPS, the substrates were first incubated in solutions containing 250 µM (~ 500 µg) of each peptide (eBGU1, eBGU3, eBGAL1, or eBGAL3) to allow for binding (as described above), then incubated in 500 µl of PBS buffer at 37 °C with constant agitation for 5 days (eBGU1 and eBGU3 peptides) or 10 weeks (eBGAL1 and eBGAL3 peptides), respectively. Whole buffer solutions were changed at indicated time points and the amount of peptide released from HPS was determined via fluorescence analysis and comparison with standards containing known amounts of 5(6)-FAM. The fluorescent images of fluorescently-labeled peptides bound to HA-coated films were obtained using an Olympus IX51 fluorescence microscope (Olympus, Center Valley, PA).

2.4. Culture of hMSCs

hMSCs (Cambrex, Walkersville, MD, passages 5–6) were cultured in mesenchymal stem cell growth medium (MSCGM: Cambrex) consisting of MSC Basal Medium supplemented with 10% fetal bovine serum, L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin and grown using culture methods described elsewhere [20]. 2.5×10^4 hMSCs were seeded onto either tissue culture-treated polystyrene (TCP) or four different types of experimental substrates (1 cm²) (PLG, HA-coated PLG, eBGU3-treated HA coating, or eBGa3-treated HA coating). hMSCs were allowed to attach to each substrate overnight, then cultured in MSCGM with osteogenic culture supplements (OS) (0.1 μ M dexamethasone, 50 μ g/ml ascorbic acid, and 10 mM β -glycerophosphate) for 24 days. The effects of soluble peptides included in culture medium were evaluated by adding 50 μ g of eBGU3 or eBGa3 peptides to hMSC cultures on TCP in 500 μ l of medium with or without osteogenic culture supplements. In each experimental and control sample, whole volume medium changes were performed every 4 days by replacement with fresh medium and collected medium was used for BMP-2 and OCN ELISA assays.

2.5. Quantification of ALP Activity

The biological activity of modular peptides was initially assayed by their ability to enhance ALP activity in hMSCs. AP Assay Reagent S (GenHunter, Nashville, TN) was used for cell staining and the EnzoLyte pNPP Alkaline Phosphatase Assay Kit (Anaspec, San Jose, CA) was used to measure enzymatic activity of ALP at day 12. For ALP staining, cells were washed with 1 ml of 1X PBS and 10% formalin, incubated at RT for 30 min, and washed again with PBS, and this wash was repeated 3 times. Cell layers were then stained with 0.5 ml of AP Assay Reagent S and incubated at RT for 30 min. Cell layers were washed 3 times with 1X PBS after staining was completed. Images of stained samples were captured via an Olympus IX-51 inverted microscope. For the ALP activity assay, cells were washed twice with a lysis buffer containing 0.1% Triton X-100. The lysate was centrifuged, and the resulting supernatant was assayed for ALP activity by incubating with 50 μ l p-nitrophenyl phosphate (pNPP) in an assay buffer at 37 °C for 15 min. ALP activity was measured at 405 nm, and calculated as the ratio of p-nitrophenol released to total DNA concentration (nmol/min/ μ g DNA). To determine the amount of total DNA in each well, the cell nuclei were disrupted by addition of the aforementioned lysis buffer followed by centrifugation, and quantified using the CyQUANT Assay Kit (Molecular Probes, Eugene, OR).

2.6. Characterization of Mineralized Tissue Formation

The influence of modular peptides on mineralized nodule formation in hMSC culture was assessed as described previously [21]. Characterization of mineralized tissue growth was performed via Alizarin Red-S (ARS) staining at day 20. The cultured cells on each type of substrate were washed with PBS and fixed in 10% (v/v) formaldehyde at RT for 30 min. The cells were then washed twice with excess dH₂O prior to addition of 1 ml of 40 mM ARS (pH 4.1) per well for 30 min. After aspiration of the unincorporated ARS, the wells were washed four times with 4 ml dH₂O while shaking for 10 min. For quantification of staining, 400 μ l 10% (v/v) acetic acid was added to each well for 30 min with shaking. The cell monolayers were then scraped from the substrates and transferred with 10% (v/v) acetic acid to a 1.5-ml tube. After vortexing for 30 sec, the slurry was overlaid with 250 μ l mineral oil, heated to 85 °C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 15,000g for 15 min and 300 μ l of the supernatant was removed to a new 1.5-ml tube. Then 200 μ l of 10% (v/v) ammonium hydroxide was added to neutralize the acid. Aliquots (100 μ l) of the supernatant were read in triplicate at 405 nm in 96-well plate reader.

2.7. BMP-2 and Osteocalcin ELISAs

Two ELISA kits were to quantify the secreted amount of BMP-2 (Quantikine BMP-2 Immunoassay, R&D Systems, Minneapolis, MN) and osteocalcin (Gla-type Osteocalcin EIA Kit, Zymed, Carlsbad, CA) in culture media according to manufacturers instructions. Cell culture media were collected from various culture conditions at days 8, 16, and 24 and then measured for BMP-2 and osteocalcin protein levels.

2.8. RNA Purification and RT-PCR Analysis

For mRNA analysis, the adherent cells were removed from culture dishes or each cultured substrate via 0.05% trypsin and resuspended in 350 μ l RLT buffer (Qiagen, Valencia, CA). Total RNA was extracted using RNeasy mini-kits (Qiagen). First-strand cDNA was synthesized from 0.5 μ g total RNA with 0.5 μ g pd(T)₁₂₋₁₈ as the first strand primer, using Ready-to-Go RTPCR Beads (GE Healthcare, Piscataway, NJ), and then amplified by PCR using primer sets (Fig. 5A) in a Robocycler Gradient 96 (Stratagene, La Jolla, CA). Cycling conditions were as follows: 97 °C for 5 min followed by 32 cycles of amplification (95 °C denaturation for 30 sec, 60 °C annealing for 30 sec, 72 °C elongation for 30 sec), with a final extension at 72 °C for 5 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with SYBR gold nucleic acid gel stain and relative gene ratios of OCN, OPN, and Cbfa1 *versus* β -actin gene were measured by densitometry.

2.9. Statistical Analysis

All data are given as mean $\pm$ standard deviation. Statistical comparisons of the results were made using one way analysis of variance (ANOVA) with Dunnett's post hoc tests. Shapiro-Wilk method was used if normality test is needed. The data analyses were performed with Statistical Program for the Social Sciences (SPSS) software and differences were considered significant at $p < 0.05$ between control and experimental groups.

3. Results

3.1 Characterization of Modular Peptides

Modular peptides consisting of a BMP2-derived sequence and a series of mineral-binding sequences inspired by OCN were synthesized via standard solid phase synthesis (Table 1). This series of peptides differs in the characteristics of the Gla residues in the OCN-inspired sequence. More specifically, the peptides contained either all three Gla residues, or contained substitutions of Gla residues with either Glu or Ala. We hypothesized that the Glu and Ala substitutions would influence the net charge and secondary structure of the modular peptides, and would therefore influence peptide-mineral binding. The two components of the peptides were separated by a (Ala)₄ sequence. The (Ala)₄ sequence was chosen as both a spacer and an extension, as the OCN-derived sequence has been shown to be α -helical in native OCN, and poly(Ala) sequences have a known propensity to form α -helices [22]. The resulting series of modular peptides were expected to be biologically active and bind to HA surface with variable affinity.

3.2 Modular Peptide Binding and Release Kinetics

We previously developed a process that allows for growth of a "bone-like" HA mineral coating on poly (lactide-co-glycolide) (PLG) substrates in simulated physiological conditions. The characteristics of these HA coatings have been detailed previously [19], and our results here corroborate previous studies. Specifically, SEM images (Figure 1A–C) and XRD spectra (Figure 1D) demonstrated that the mineral layer grown on the PLG surface had a plate-like nanostructure and a HA phase, similar to vertebrate bone mineral in structure and composition.

The binding efficiency of modular peptides on the HA-coated PLG substrates (HPS) was sequence-dependent and increased in the following order: eBGu3 ($7.6 \pm 7.8\%$) < eBGu1 ($10.3 \pm 4.7\%$) < eBGa1 ($29.9 \pm 2\%$) < eBGa3 ($55.9 \pm 2.2\%$) (Figure 2A). The binding efficiency of eBGa3 was substantially higher than other peptides studied ($p^{*†} < 0.005$), and we therefore studied the binding of this molecule in detail. The amount of bound eBGa3 on the HPS increased with peptide concentration and reached saturation at approximately $150\ \mu\text{M}$ ($300\ \mu\text{g}$) (Figure 2B). The release kinetics of the modular peptides from HA-coated substrates were also highly dependent on the mineral-binding sequence (Figure 2C and D). eBGu1 ($98.89 \pm 18.84\%$ after 5 days) and eBGu3 ($93.33 \pm 17.24\%$ after 5 days) peptides were released rapidly from HPS surfaces. In contrast, the eBGa3 peptide was released much more slowly, as only $15.7 \pm 0.6\%$ of peptide was released after 70 days (Figure 2D). Notably, these data indicate that nearly 85 % of the initially bound eBGa3 peptide remained bound after 70 days.

3.3 Biological Activity of Modular Peptides

Soluble modular peptides added to hMSC growth medium along with osteogenic supplements had a positive influence on osteogenic differentiation of hMSCs. Specifically, the eBGa3 peptide significantly increased ALP activity ($p = 0.017$) (Figure 3A) and mineralized tissue formation ($p = 0.018$) (Figure 3B). Importantly, there were no significant differences between the positive effects of eBGu3 and eBGa3 when added as soluble supplements to standard hMSC culture, suggesting that the biological activity of the BMP2-derived portion of the peptides was not significantly influenced by the sequence of the HA-binding portion.

When bound to a HA-coated substrate the eBGa3 peptide significantly enhanced ALP activity and mineralized tissue formation by hMSCs (Figure 4A and B). hMSCs cultured on eBGa3-bound, HA-coated substrates (termed “HeBGa3 substrates”) expressed significantly higher ALP activity ($0.48 \pm 0.06\ \text{nmol/min}/\mu\text{g DNA}$) than hMSCs on untreated TCP (0.25 ± 0.02), PLG (0.30 ± 0.02), or HA-coated (0.30 ± 0.03) substrates (Figure 4A). Similarly, Alizarin red S staining of mineralized tissue was significantly increased on HeBGa3 substrates ($4.32 \pm 0.57\ \text{mM/well}$) when compared to untreated TCP (0.76 ± 0.12), PLG (0.98 ± 0.14), or HA-coated (1.66 ± 0.6) substrates (Figure 4B). Importantly, HeBGa3 substrates also induced enhanced BMP-2 secretion (Figure 4C, days 16 and 24) and OCN production (Figure 4D, days 8, 16, and 24) when compared to untreated substrates. Specifically, the hMSCs cultured on HeBGa3 produced a 6-fold higher amount of BMP-2 protein ($311.59 \pm 94.55\ \text{pg/ml}$) when compared to TCP ($43.36 \pm 18.60\ \text{pg/ml}$) at day 24 ($p = 0.002$) (Figure 4C), and OCN production was approximately 3-fold higher on HeBGa3 substrates ($172.98 \pm 5.7\ \text{ng/ml}$) when compared to TCP substrates ($60.21 \pm 10.62\ \text{ng/ml}$) on day 8 ($p < 0.0001$) (Figure 4D). Taken together, these data indicate that the eBGa3-treated substrates promote osteogenic differentiation of hMSCs.

The effects of eBGu3-treated, HA-coated substrates (termed “HeBGu3 substrates”) on osteogenic differentiation of hMSCs were less pronounced than the effects of the HeBGa3 substrates. Specifically, HeBGu3 substrates did not significantly enhance ALP activity of hMSCs (Figure 4A), but did significantly enhance mineralized tissue formation ($p < 0.02$) (Figure 4B). Effects of HeBGu3 substrates on production of BMP2 and OCN were significant at day 8 and day 16, but not significant at day 24. These data indicate that the eBGu3-treated substrates can promote osteogenic differentiation of hMSCs, but the effects are not as substantial as the effects of eBGa3-treated substrates.

3.4. Expression of Osteogenic Markers

We next focused on correlating osteogenic differentiation with the expression levels of osteogenesis-related proteins, including OCN, osteopontin (OPN), and core-binding factor alpha 1 (Cbfa1) via RT-PCR using the primers indicated (Figure 5A). OCN expression was significantly increased on HeBGa3 substrates at all time points studied when compared to TCP

($p < 0.01$), PLG ($p < 0.01$), and HPS ($p < 0.04$) (Figure 5B and C). OPN expression was significantly increased on HeBGa3 substrates at day 8 ($p = 0.005$) and day 16 ($p = 0.032$) when compared to TCP (Figure 5B and D). Cbfa1 expression was increased on HeBGa3 substrates at all time points studied when compared to TCP ($p < 0.002$) (Figure 5B and E). Expression of osteogenesis-related genes was also enhanced on HeBGu3 substrates compared to TCP, PLG, and HPS, but to a lesser extent than HeBGa3 substrates. Specifically, HeBGu3 substrates enhanced OCN expression at day 8 and enhanced Cbfa1 expression at all time points studied. Taken together, the RT-PCR analyses indicate that eBGa3-treated substrates promote expression of osteogenic markers to a greater extent than eBGu3-treated substrates, and this result is in agreement with the aforementioned analyses of ALP activity, mineralized tissue formation, BMP-2 production, and OCN production. It is noteworthy that Cbfa1 expression was also enhanced on HA-coated substrates when compared to TCP at day 16 ($p = 0.031$) and day 24 ($p < 0.044$), indicating that the HA substrate alone slightly influences expression of pro-osteogenic transcription factors.

4. Discussion

In the present study we developed modular peptides designed to bind to an HA-coated surface and retain the biological activity of BMP-2. Specifically, the modular peptides each included a short sequence inspired by N-terminal portion of the protein OCN, which served as a linker to immobilize a 20 amino acid BMP2-derived sequence. The sequence of the OCN-inspired portion within the peptides was varied (see Table 1) to modulate their level of binding to a HA-coated surface as well as their release kinetics from the substrate.

The mineral surfaces formed in the present study are similar to bone mineral in structure and composition. The surface was composed of a poorly crystalline HA mineral phase with a plate-like morphology at the nanometer scale (Figure 1). Previous studies using these types of coatings on PLG films indicated that the mineral is a carbonate-substituted HA, which degrades over time in aqueous solution [18,23]. Each of these characteristics is consistent with those of the major mineral component in vertebrate bone tissue, and these types of coatings have therefore been termed “bone-like mineral” coatings. Importantly, the process used to form these mineral coatings can be generalized to a broad range of biomaterials. Previous studies by our group and several others have generated bone-like apatite coatings on biodegradable plastics, metals, glasses, and hydrogels using biomimetic approaches similar to the one described here (reviewed by Kokubo et al. in [24]). Therefore, each of these types of materials can potentially be HA-coated and used as a platform for incorporation of the modular peptides developed in our current study.

The eBGa3 peptide bound most strongly to HA-coated substrates, indicating that this peptide molecule mimicked the ability of OCN to bind to HA surfaces. This result is consistent with previous studies, which have shown that Gla residues play an important role in binding of native OCN [25] and *de novo* designed peptides [26] to HA surfaces. In addition, substitution of Gla residues with either Ala or Glu resulted in significant decreases in binding efficiency (Figure 2A). These changes in peptide-mineral interaction may be attributed to changes in the charge density and secondary structure of the modular peptides. Gla residues contain two anionic carboxyl groups per side chain while Glu and Ala residues contain one or none, respectively. In addition, the previous studies noted above indicate that Gla residues play a vital role in formation of α -helical secondary structure at the N-terminus of native OCN [25] and in a *de novo* designed Gla-rich peptide [26]. Although the secondary structure of peptides was not examined here, we hypothesize that the eBGa3 peptide may adopt α -helical structure upon binding to HA-coated substrates, which may contribute to their high binding efficiency.

Importantly, modular peptides in which Gla residues were replaced with either Glu or Ala residues were released from the HA-coated substrates more rapidly when compared to eBGa3 (Figure 2C and D). These results demonstrate that immobilization and subsequent release of peptides can be tailored by choosing the appropriate mineral-binding sequence from the listing in Table 1. Rapidly released peptides such as eBGu3 (Figure 2C and E) may ultimately be useful to influence physiological processes that occur over short timeframes (e.g. acute inflammation, early angiogenesis). In contrast, sustained presence of peptides such as eBGa3 (Figure 2D and F) may ultimately be useful to influence long-term processes (e.g. tissue morphogenesis).

Modular peptides were biologically active when presented to hMSCs either in solution or immobilized on HA-coated substrates. This finding is generally consistent with previous approaches that have used covalent reactions to immobilize BMP-2. Liu et al. recently immobilized full-length BMP-2 to PEG hydrogels [27] and showed increased osteogenic MSC differentiation. Becker et al. covalently-linked full-length BMP-2 to titanium implants and showed enhanced appositional bone growth in a canine model [28]. In addition, the BMP-2-derived 20-mer peptide explored in our current study has been covalently linked to alginate hydrogels [14] and titanium substrates [29] for enhanced ectopic bone formation or osteogenic differentiation, respectively. These previous studies along with the data presented here strongly indicate that BMP-2 remains biologically active when immobilized on a biomaterial. Therefore, the approach developed here could potentially be used to confer BMP2's biological activity onto the wide range of HA materials and coatings used commonly in orthopedic applications.

The effects of the eBGu3 peptide on osteogenic differentiation of hMSCs were less pronounced when compared to effects of the eBGa3 peptide. This decreased effect can be attributed to decreased exposure of hMSCs to eBGu3 peptide molecules. More specifically, the amount of eBGu3 molecules bound to the HA-coated surface at the outset of the experiments was significantly lower than the amount of eBGa3 molecules due to decreased binding efficiency (Figure 2A). In addition, the more rapid release of the eBGu3 peptide molecules when compared to minimal release of eBGa3 molecules (Figure 2C and D) indicates that hMSCs were exposed to eBGu3 molecules for a shorter timeframe, and we hypothesize that this decreased exposure time also led to a decrease in the level of induced osteogenic differentiation. This hypothesis is supported by previous work by Puleo and coworkers, which indicates that the time duration of exposure to full-length BMP-2 protein influences osteogenic induction of hMSCs, with longer exposure times resulting in more substantial osteogenic induction [30]. It is less likely that the enhanced osteoinductive effects of eBGa3 peptide are due to intrinsic differences in biological activity of eBGa3 versus eBGu3, as these two peptides have similar effects on hMSC differentiation when presented in solution rather than on the HA-coated surface (Figure 3).

Recent studies indicate that mRNA levels of OCN, OPN, and Cbfa1 are up-regulated during osteogenic differentiation of hMSCs *in vitro* [10,31]. Similarly, our results demonstrated that expression levels of each of these markers significantly increased on eBGa3-treated substrates. The temporal expression profiles of these osteogenic markers differed from one another. While OCN expression gradually increased over 24 days in culture, OPN expression decreased from day 8 to day 24 (Figure 5). Similarly, previous reports indicate that up-regulated expression of OPN is related to early osteogenic differentiation of MSCs [32–36], while up-regulated expression of OCN is related to mineralized tissue formation by MSCs during later stages of differentiation. Therefore, the temporal pattern of osteogenesis-related gene expression observed in this study is consistent with previous reports of osteogenic differentiation of MSCs *in vitro*.

The osteogenic differentiation process promoted by modular peptides in the present study had similar characteristics when compared to MSC differentiation promoted by soluble BMP-2 protein in previous studies. Diefenderfer et al. demonstrated that BMP-2 alone is not sufficient to induce enhanced ALP activity by hMSCs but that soluble BMP-2 promotes hMSC differentiation when presented to cells after pre-treatment with dexamethasone [37]. Similarly, our results indicate that soluble modular peptides presented to cells in conjunction with osteogenic supplements (OS) media, including dexamethasone, promoted enhanced expression of bone markers when compared with OS media alone (Figure 3). Importantly, the effects of the eB3Gu3 and eB3Ga3 peptides were more pronounced when they were presented on an HA-coated substrate (Figure 4–5) rather than simply added as soluble components to hMSC culture medium (Figure 3). Therefore, surface immobilization enhances the effects of these peptide molecules. This effect may be due to the increased exposure time of cells to modular peptide molecules, as noted above.

5. Conclusion

We designed modular peptides to combine the mineral-binding capability of osteocalcin with the biological activity of BMP-2. These modular peptides were capable of binding to hydroxyapatite coatings formed on biodegradable poly(lactide-co-glycolide) films. Binding to hydroxyapatite-coated substrates, and subsequent release, could be varied significantly by changing the sequence of the OCN-inspired, hydroxyapatite-binding portion. The BMP2-derived peptide sequence in the modular growth factors was capable of promoting osteogenic differentiation when presented to hMSCs in solution or immobilized on a hydroxyapatite coating, indicating that the BMP2-derived peptide sequence remained biologically active. Although here we focus specifically on a BMP2-mimetic modular peptide to promote stem cell differentiation, a variety of small molecules, peptides, or proteins can potentially be incorporated onto various natural HA surfaces or HA-based biomaterials using this general approach. Therefore, the approach described here may represent an enabling strategy not only in stem cell-based regenerative medicine but also as a pharmaceutical agent in treatment of degenerative skeletal diseases (e.g. osteoporosis) or as a probe for detection of diseases associated with aberrant calcification (e.g. atherosclerosis, cancer).

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Figure 1.

Bone-like HA coatings can be grown on PLG substrates using a biomimetic approach. SEM images of a PLG film (A) and a mineral-coated PLG film (B) (scale bars - 20 μm). (C) High magnification SEM image of a HA-coated substrate, demonstrating plate-like nanoscale morphology (scale bar - 1 μm). (D) X-ray diffraction spectrum of PLG substrates and HA-coated PLG substrates (* denotes peaks associated with HA mineral phase).

Figure 2.

Modular peptide binding and release kinetics are sequence-dependent. (A) Binding efficiency of various 5(6)-FAM tagged modular peptides in PBS containing 500 μg of each peptide. Each modular peptide amount on the HPS substrate was expressed as percent of peptide mass bound to the surface relative to the initial mass of peptide present in the solution. Symbols * and ‡ indicate statistically significant differences when compared to eBGu1 or eBGa1 peptides, respectively (p < 0.01). (B) Binding isotherm of eBGa3 peptide on HA-coated substrates. HPS were incubated in various concentrations (50–750 μM) of 5(6)-FAM-conjugated eBGa3 peptide at 37 °C for 4 h. (C–D) Cumulative release curves of eBGu1 and eBGu3 peptides over 5 days (C) and eBGa1 and eBGa3 peptides over 70 days (D). The data are presented in terms of the percent of peptide mass released compared to the initial mass of peptide bound. (E and F) Fluorescence micrographs of HPS after release of 5(6) FAM-labeled peptides from HPS surface for various timeframes. The bottom of each image schematically represents bound peptides on HA-coated substrates. (G) Surface fluorescence images of eBGa1 and eBGa3 peptides immobilized onto HPS after 10 weeks of release.

Figure 3.

Osteogenic activity of modular peptides. (A) ALP activity of hMSC cultured in the presence of soluble modular peptides in the presence or absence of osteogenic supplement (left), and ALP staining of hMSC cultured with soluble modular peptides in the presence of osteogenic supplement medium at day 12 (right). 50 μg of modular peptides were added to hMSC cultures on TCP in 500 μl of medium/well. Whole volume medium changes were performed every 4 days by replacement with fresh medium containing 50 μg of modular peptide. (B) ARS assay of hMSCs cultured in the presence of soluble modular peptides with or without osteogenic supplements (left), and staining (calcium deposition) of hMSCs cultured in the presence of soluble modular peptides with osteogenic supplement medium at day 20 (right). * indicates statistically significant differences ($p < 0.05$) compared with control (TCP) in the presence of osteogenic supplement medium. Osteogenic supplement includes 0.1 μM dexamethasone, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. OS (-) represents conditions without osteogenic supplement.

Figure 4.

Biological activity of modular peptides on HA-coated substrates. (A) ALP activity of hMSCs was characterized by measuring synthesized p-nitrophenyl phosphate (pNPP) at day 12. (B) Mineral deposition was determined by Alizarin Red S (ARS) staining at culture day 20. (C and D) BMP-2 and OCN protein in the culture media were measured by ELISA at each indicated time point. hMSC are cultured in the presence of osteogenic supplement in all experiments. * indicates $p < 0.05$ when eBGU3 treatment or eBGa3 treatment was compared to three different untreated culture conditions (TCP, PLG, and HA-coated PLG).

Figure 5.

mRNA expression levels of hMSCs measured by RT-PCR. (A) Description of the designed primers. RT-PCR analysis was performed on total RNA at the indicated time points. (B) Densitometry of the osteogenic-related genes was compared with β -actin as an internal reference. A representative gel is shown for day 8 of culture. (C, D, and E) The PCR products were analyzed by electrophoresis and relative gene ratios of OCN, OPN, or Cbfa1 *versus* the β -actin gene was compared and represented in each graph (C, D, and E). hMSC are cultured in the presence of osteogenic supplement in all experiments. * indicates statistically significant differences ($p < 0.05$) compared with control (TCP) at each time point.

Table 1

Amino acid sequence of modular peptides and natural templates

Abbreviation	Amino Acid Sequence (definition)
hBMP-2	KIPKACCVPTELSAISMLYL (AAs: 73–92)
hOCN	γ EP RR γ EV C γ EL (AAs: 17–25)
^a eBMP2	KIPKASSVPTELSAISTLYL (engineered BMP2 peptide)
eBGa3	KIPKASSVPTELSAISTLYL-AAAA- γ <u>EP</u> RR γ <u>EV</u> A γ <u>EL</u>
eBGa1	KIPKASSVPTELSAISTLYL-AAAA- γ <u>EP</u> RR <u>A</u> V <u>A</u> <u>A</u> <u>L</u>
eBGu3	KIPKASSVPTELSAISTLYL-AAAA- <u>EP</u> RR <u>E</u> V <u>A</u> <u>E</u> <u>L</u>
eBGu1	KIPKASSVPTELSAISTLYL-AAAA- <u>EP</u> RR <u>A</u> V <u>A</u> <u>A</u> <u>L</u>
HeBGa3	HPS:KIPKASSVPTELSAISTLYL-AAAA- γ <u>EP</u> RR γ <u>EV</u> A γ <u>EL</u> (HA-coated PLG substrate treated with eBGa3)
HeBGu3	HPS:KIPKASSVPTELSAISTLYL-AAAA- <u>EP</u> RR <u>E</u> V <u>A</u> <u>E</u> <u>L</u> (HA-coated PLG substrate treated with eBGu3)

^a eBMP-2 peptide sequence was originally synthesized by Tanihara and coworkers.

Note: γ E represents γ -carboxylated glutamic acid (Gla).

Note: amino acids that are varied in modular peptides are underlined.