IN VITRO MINERALIZATION STUDIES WITH SUBSTRATE-IMMOBILIZED BONE MORPHOGENETIC PROTEIN PEPTIDES

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KEY WORDS

Osteoblast
BMP-7 peptides

Understanding the factors that control osteoblastic behavior is centrally important in establishment of successful osseointegration. Pharmacogenetic control of the osteoblast to increase the mineral content around dental implants may offer a unique advantage to clinicians in improving osseointegration success and decreasing time before mechanical loading. This in vitro pilot study has screened for bioactive peptides derived from bone morphogenetic protein 7 (BMP-7) (also called osteogenic protein 1 [OP-1]). Thirteen overlapping peptides of BMP-7 were synthesized and covalently coupled to glass coverslip substrates using silane chemistry. The rate and relative amount of mineralization were compared by von Kossa analysis using primary rat calvarial osteoblastic cell populations during a 7- to 21-day period. In addition, bone sialoprotein (BSP) and osteocalcin (OC) gene expression was measured from osteoblastic cells grown on peptide-immobilized glass coverslips by reverse transcriptase–polymerase chain reaction. Initial results from mineralization studies suggested the BMP-7–derived peptides were able to support mineralization to varying degrees with enhanced peptide-induced mineralization from the C- and N-termini of the BMP-7 molecule. Analysis of these peptide regions indicated that these peptides comprised the finger 1 and 2 domains of OP-1, which contribute toward ligand-receptor interaction. Further analysis of gene expression from select peptide-immobilized substrates indicated that peptides from the C-terminus of BMP-7 were capable of supporting BSP and OC messenger RNA expression. These studies indicate that BMP-7 peptides covalently bound to solid substrates may provide the biological basis to immobilize peptides to titanium implants to induce osteoblastic differentiation and mineralization in a more predictable fashion.

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INTRODUCTION

The boundary between implant materials, including titanium, and tissues can be strengthened by coating these substrates with various molecules that attract, bind, and activate specific cell types to behave in a predictable fashion.[1] Throughout the years, many surface modifications have been developed to encourage direct bone apposition to titanium surfaces. Osteoblast differenti-

TABLE 1

Sequences of BMP-7–derived peptides*

Peptide No.	Sequence	Peptide No.	Sequence
1	STGSKQRSQN RSKTPKNQEA	8	CAFPLNSYMN ATNHAIVQTL
2	RSKTPKNQEA LRMANVAENS	9	ATNHAIVQTL VHFINPETVP
3	LRMANVAENS SSDQRQACKK	10	VHFINPETVP KPCCAPTQLN
4	SSDQRQACKK HELYVSFRDL	11	KPCCAPTQLN AISVLYFDDS
5	HELYVSFRDL GWQDWIIAPE	12	AISVLYFDDS SNVILKKYRN
6	GWQDWIIAPE GYAAYYCEGE	13	SNVILKKYRN MVVRACGCH
7	GYAAYYCEGE CAFPLNSYMN		

*The mature form of the human bone morphagenic protein 7 (BMP-7) osteogenic protein-1 (OP-1) sequence (NCBI accession No. P18075) was divided into 13 different peptides that were 15 amino acids in length and that overlapped another peptide by 10 sequences. The resulting peptides (designated 1 through 13) were used in the present study. The BMP-7 protein sequence is as follows:

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1   STGSKQRSQN RSKTPKNQEA LRMANVAENS SSDQRQACKK HELYVSFRDL GWQDWIIAPE
61  GYAAYYCEGE CAFPLNSYMN ATNHAIVQTL VHFINPETVP KPCCAPTQLN AISVLYFDDS
121 SNVILKKYRN MVVRACGCH
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TABLE 2

Amplification primer sets used in polymerase chain reaction*

Primer	Sequence (5′-3′)	Expected base pairs	Accesion No.
GAPDH—sense (+)	CACCATGGAGAAGGCCGGGG	418	NM023964
GAPDH—antisense (−)	GACGGACACATTGGGGGTAG		
OC—sense	TCTGACAAACCTTCATGTCC	198	NM013414
OC—antisense	AAATAGTGATACCGTAGATGCG		
BSP—sense	AACAATCCGTGCCACTCA	1068	NM012881
BSP—antisense	GGAGGGGGCTTCACTGAT		

*GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase; OC, osteocalcin; and BSP, bone sialoprotein. GenBank accession Nos. are provided for DNA sequences used in reverse transcriptase–polymerase chain reation.

ation and mineralization have been demonstrated on titanium, glass, and hydroxyapatite-coated implant surfaces.[2,3] Using various methods, including immunocytochemical analysis and electron microscopy, no significant differences have been observed between implant surface coatings and mineralization in tissue culture.[4] Thus, more recent experimental strategies have focused on recombinant protein use to enhance bone formation around titanium implants.

Several growth factors have been used to induce bone formation around titanium implants. These include combinations of insulinlike growth factor and platelet-derived growth factor in a beagle dog model,[5] transforming growth factor β1 (TGF-β1) when applied to titanium surfaces,[6] and basic fibroblast growth factor to increase angiogenesis in the area adjacent to the implant to increase subsequent bone formation.[7] However, the most potent inducers of ectopic bone formation around titanium implants described to date are members of the bone morphogenetic protein (BMP) family of protein growth factors.[8,9] The osteogenic capacity of many BMPs has been proven by implanting individual recombinant BMPs in a rat ectopic assay and using stringent criteria. BMP 2, 4 through 7, and 9 have been shown to be bone inductive molecules.[10] Moreover, both recombinant osteogenic protein 1 (OP-1) and partially purified osteogenic protein preparations have been shown to enhance osseointegration around dental implants.[11,12]

Several studies during the past few years have focused on the role of BMPs during osteoblast differentiation. Osteoblasts express various phenotypic markers, such as alkaline phosphatase and synthesize collagenous and noncollagenous bone matrix proteins, including osteocalcin (OC).[13] Osteoblast differentiation is regulated by many local factors in a paracrine and/or autocrine fashion.[14] BMP-7 (or OP-1) has been shown to increase alkaline phosphatase activity, parathyroid hormone responsiveness, and OC production in osteoblastic model systems,[2,13–15] suggesting that BMP-7 stimulated osteoblast differentiation from precursor cells.

Covalent chemical strategies are often used to modify biomaterial surfaces with bioactive compounds. The rationale of these strategies has been supported by previous studies that showed that molecules covalently immobilized on surfaces are retained more dependably than molecules that are simply adsorbed.[1,16,17] The use of biomimetic engineered surfaces have mainly used adhesive peptides, including argine-glycine-aspartate or RGD peptide sequences, which mimic integrin receptors.[18–21] These peptides have been used either adsorbed or covalently attached on different substrates, including titanium. Relatively fewer studies have used BMP-derived peptides to determine if these peptides are osteogenic. A recent study has used an expressed BMP-2 peptide containing 102 amino acids of the C-terminal end of the molecule to induce bone formation,[22] and others have studied the osteogenic growth peptide, which is a positively charged 14-amino acid growth polypeptide generated as a posttranslational cleavage product using an alternative translational initiation codon of the histone H4 gene.[23–25] Therefore, the present study sought to identify the minimal bioactive sequences from BMP-7 for potential immobilization on biomaterials and use in control of osteoblast functions.

METHODS AND MATERIALS

Peptide design and synthesis

Thirteen overlapping peptides of 20 amino acids each (Table 1) derived

from the mature form of human BMP-7 molecule (amino acid residues 293–431, National Center for Biotechnology Information accession No. P18075) were custom synthesized by and purchased from American Peptide Company Inc (Sunnyvale, Calif).

Substrate preparation

Peptides were covalently immobilized on borosilicate glass coverslips (Fisher Scientific, Pittsburgh, Pa) using established techniques.[19,20] Briefly, substrates were treated with 2% 3-aminopropyltriethoxysilane and 2% triethylamine in acetone (chemicals from Sigma Chemical Company, St Louis, Mo) under an argon environment to yield ''aminated'' substrates or substrates covered with a polymerized silane layer that possessed functional amine groups. Peptides were then covalently bound to the aminated substrates during incubation with a 25:25:1 (vol/vol/vol) solution of peptide (0.1 mM in dry *N,N*-dimethylformamide), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (2.5 mg/mL^{-1} in dry *N,N*-dimethylformamide), and *N*-ethyl morpholine (all chemicals from Sigma). Adsorbed peptides were removed using 4 M urea and 1 M sodium chloride. Control substrates used in the present study included plain and aminated glass coverslips. All substrates were stored under argon or nitrogen until sterilization (via immersion in 70% ethanol) and use in cell culture experiments.

Cell culture of primary rat calvarial cells

Osteoblast-enriched cell preparations were obtained from Sprague-Dawley 21- or 28-day fetal rat calvaria by sequential collagenase digestion (Type II, Invitrogen Life Technologies, Carlsbad, Calif) in bone cell buffer, pH 7.4, as described previously.[26] The resultant cells from the third and fourth 15-minute collagenase digestions have osteoblastic character, including high alkaline phosphatase activity and the ability to form collagen and bone in vitro.[26] These cells were pooled and cultured in BGJb media (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air.

Mineralization assay

Primary rat calvarial cells were seeded in 6-well dishes containing peptide-immobilized or control coverslips at an initial cell density of 2500 cells per well. On confluency, the media was changed to include supplements of β-glycerophosphate (10 mM, Sigma) and L-ascorbate (50 μg/mL, Sigma). Exogenously added human recombinant BMP-7 (50 μg/mL), received as a gift from D. Rueger, Stryker Biotech Corporation (Hopkinton, Mass), was used as a positive control in these studies. Media was changed every 2 to 3 days. After 14 and 21 days of culture, the cells were fixed in 4% formalin in sodium phosphate buffer and stained for calcium salts using the von Kossa method. Digital images of the fixed and stained cultures were acquired using a Sony CCD-3 camera and a Nikon SMZ-U stereomicroscope at ×5 magnification. Optimas image-processing software (Media Cybernetics, Carlsbad, Calif) was used to detect and quantify darkly stained (mineralized) regions of the culture surface areas. One-way analysis of variance was used to determine whether the culture conditions (ie, type of substrate) affected the degree of mineralization.

Total RNA isolation of primary rat calvarial cells

Primary rat calvarial cells were plated into 6-well dishes containing coverslips with immobilized peptides or control coverslips. Total RNA from peptide-immobilized and control osteoblast cell populations was isolated using Trizol reagent (Life Technologies) 14 days after confluency in mineralizing media. Total RNA was visualized for intactness by ethidium bromide staining following gel electrophoresis. RNA was quantitated by spectroscopy (SmartSpec 3000, BioRad, Hercules, Calif).

Reverse transcription–polymerase chain reaction analysis

A total of 5 μg of total RNA was used for complementary DNA synthesis with oligo (dT)12–18 primer and Superscript II (RnaseH-) (Life Technologies) in reverse transcription (RT) reactions. A total of 2 μL of the RT product was used as a template for polymerase chain reaction (PCR) amplification of BSP, OC, and GAPDH gene products (Table 1). Standard PCR conditions were used. Semiquantitative comparison with GAPDH, an unregulated housekeeping gene, was made to assess changes in gene expression as a function of peptide treatment on agarose gels. The PCR results were quantitated using BioRad's PhosphorImager system and their Molecular Analyst software version 1.5 to assess relative differences. The RT-PCR products were assessed by subcloning RT-PCR products into pGEM-T (Promega) and DNA sequencing to determine authenticity.

RESULTS

Representative samples of mineralization are shown in Figure 1. Calcium deposition was confirmed by EDX analysis (data not shown). In Figures 2 and 3, we show mineralization data from 14- and 21-day periods normalized to tissue culture plastic (TCP). Variable induction on mineralization occurred on BMP-7–immobilized substrates at both 14-and 21-day periods. One-way analysis of variance confirmed that after 14 days, the type of cell culture substrate affected ($P = .056$, which does not meet the commonly used significance criteria of $P < .05$ but is still appropriate to report) the degree of mineralization exhibited by osteoblastic cells. After 21 days, this effect was no longer evident ($P > .10$). The analysis of variance is used to determine whether a general experimental factor (in this case, the varied surfaces of the coverslips) affected a mea-

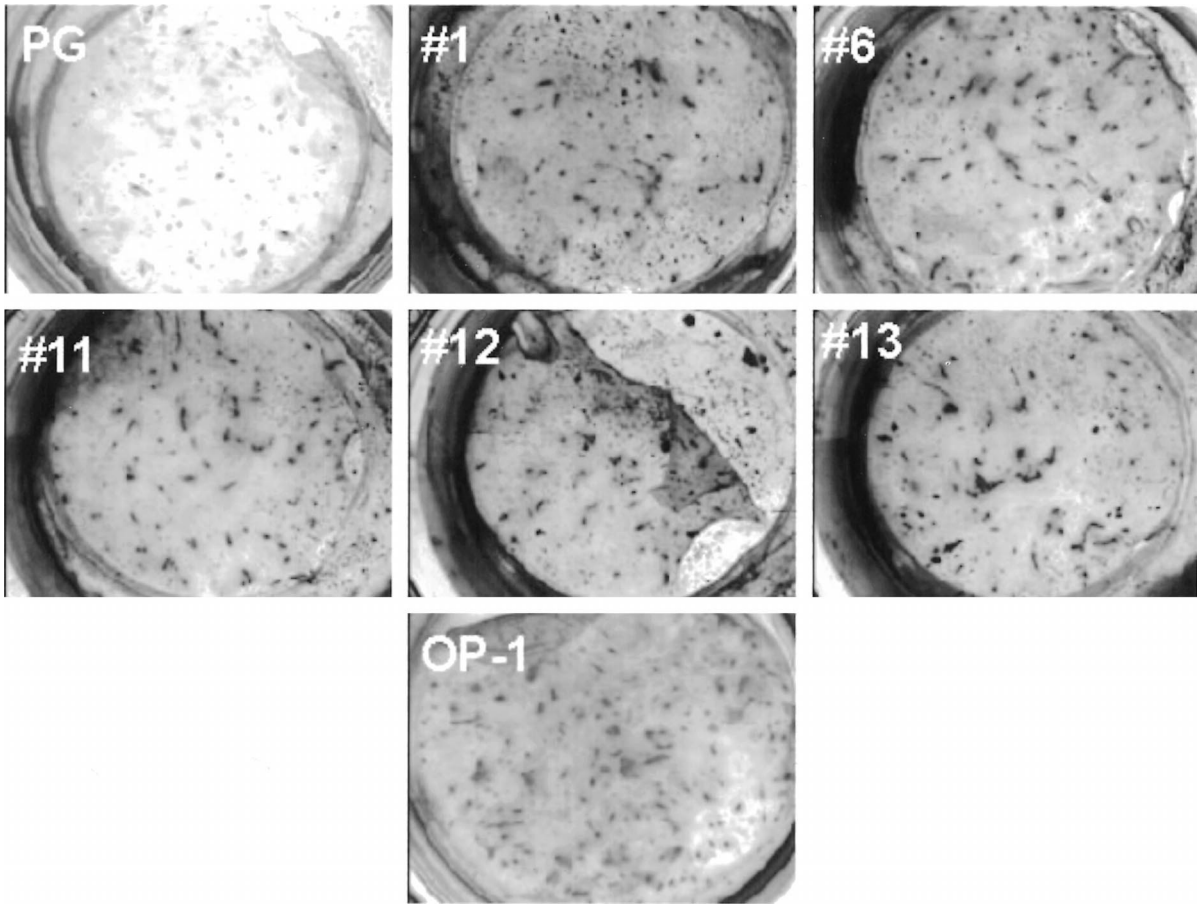


FIGURE 1. Representative in vitro mineralization data obtained from 21-day cultures of primary rat calvarial cells grown on osteogenic protein 1–immobilized peptides. Indicated peptide numbers correspond to peptides listed in Table 1. Mineralization was determined by von Kossa staining. Photographs were taken at 5× magnification.

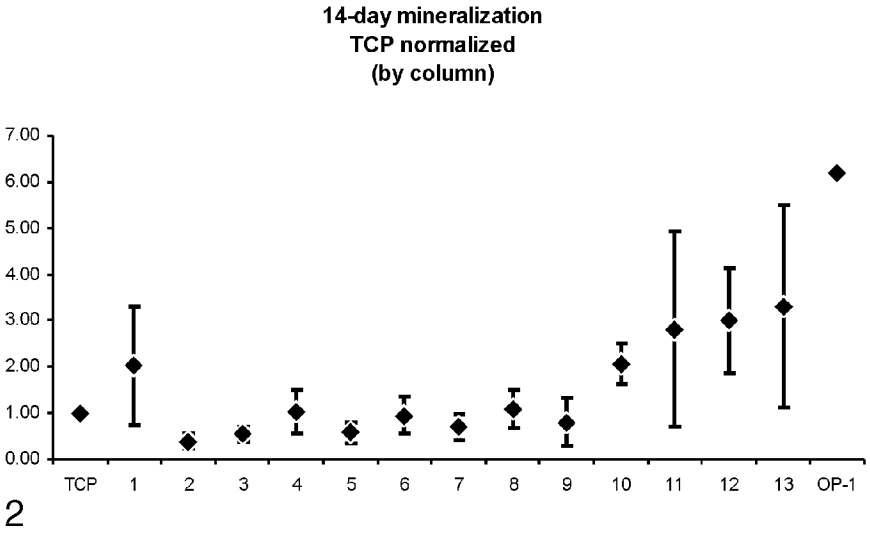
sured result not to compare the effects of individual treatments (ie, to compare the effect of one peptide to another). Statistical comparison of individual treatments can be accomplished by a number of post hoc techniques, for example, a *t* test. Although the variance within observed responses was too great for *t* tests to confirm significant differences between the amount of mineralization on substrates modified with any one peptide vs another, a trend toward enhanced mineralization was observed on peptides for the N-terminus (1) and toward the C-terminus (11, 12, and 13). These experiments were performed in duplicate to triplicate with 3 separate experiments.

From this initial screening, 5 candidate BMP-7 peptides were used in subsequent studies of gene expression. Using RT-PCR analysis, peptides 1, 6, 11, 12, and 13 were used to evaluate the potential of these peptides to induce or support messenger RNA expression of osteoblastic-specific genes associated with noncollagenous bone matrix formation, namely bone sialoprotein (BSP) and OC. In Figure 4, BMP-7, used as a positive control, was able to induce OC expression compared with controls of plain glass. In addition, all immobilized BMP-7 peptides and controls were able to support BSP expression, but only peptides 6 and 11 were able to support OC expression compared with controls after 14 days.

DISCUSSION

Design strategies for creating biomimetic materials have been the focus of several investigators in the field of tissue engineering. The common theme in engineering cell and tissue behavior at biomedical device surfaces has been to alter the surface of the material to selectively interact with specific cell types through molecular recognition signals. Thus, one of the initial steps in designing bioengineered surfaces is the molecular component of the material. In the present study, our central objective was to determine if human BMP-7–derived peptides had the ability to support or induce biomineralization through in vitro studies. BMP-7 is a well-known member of the TGF-β superfamily capable of inducing bone formation and osteoblastic cellular differentiation and secretion of bone matrix proteins.

The mature chain of human BMP-7,



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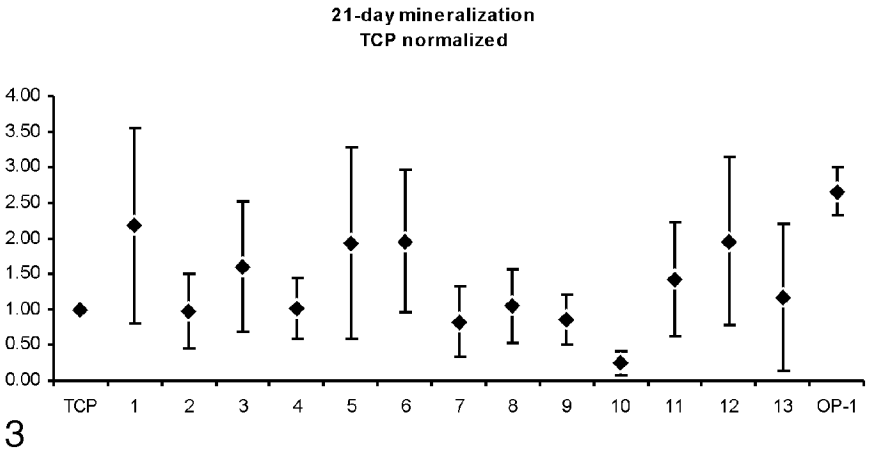

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FIGURES 2 AND 3. FIGURE 2. The 14-day mineralization experiments on osteogenic protein 1 (OP-1)–immobilized peptides. Primary rat calvarial cells were grown on immobilized peptides for 14 days after confluency. Plain glass (PG), tissue culture plastic (TCP), amino phased glass (APG), peptides 1 through 13, and OP-1 (50 μg/mL) control were evaluated. FIGURE 3. The 21-day mineralization experiments on OP-1–immobilized peptides. Primary rat calvarial cells were grown on immobilized peptides for 14 days after confluency. The PG, TCP, APG, peptides 1 through 13, and OP-1 (50 μg/mL) control were evaluated.

lacking the signal and propeptide sequences, was used in this study. Using the linear sequence, as shown in Table 1, BMP-7 was divided into 13 peptides, each of 20 amino acids in length and overlapping by 10 amino acids. Other studies had shown that peptides from BMP-2 containing 102 amino acids were capable of inducing in vivo bone formation.[22] If a minimal linear sequence was sufficient for BMP-7–induced osteogenesis activity, then tissue engineering strategies involving surface modification would be readily applicable for biomedical devices.

Covalent strategies in tissue engineering have been used to immobilize biomolecules on various surfaces to control cellular behavior. Adhesive peptides containing the Arg-Gly-Asp (RGD) sequence have been the most widely studied to date.[19,28,29] The RGD sequence mediates attachment of many types of cells, including osteoblasts, and is found in many cell membrane and extracellular matrix proteins, including fibronectin, type I collagen, osteopontin, and bone sialoprotein.[30] Other non-RGD peptides have also been examined alone or in combination with RGD peptides.[20,31-35] More recently, immobilized proteins, such as BMP-4, have been successfully immobilized onto solid substrates to modify cellular functions, including proliferation and differentiation.[36] The present study has used BMP-7 (OP-1)–derived peptides with rat calvarial osteoblastic cells to evaluate in vitro mineralization.

The primary rat calvarial cell model is a well-established in vitro model of osteoblast differentiation and mineralization. Osteoblasts isolated from the calvaria of 21-day fetal rats differentiate in tissue culture by the production and deposition of collagen-based matrix and the appearance of nodules consisting of multiple layers of cells within a mineralized extracellular matrix. Using this model system, we evaluated BMP-7 peptides once covalently attached to glass substrates for the ability to induce or support osteoblastic differentiation as measured by nodule formation and noncollagenous bone matrix gene expression. As shown in Figures 2 and 3, at both 14- and 21-day periods, peptides 6, 11, 12, and 13 were consistently able to support mineralization compared with controls. In addition, OP-1 was able to induce mineralization consistently greater than any peptides tested. Moreover, our results with mineralized nodule formation were supported by studies that addressed gene expression. As shown in Figure 4, following 14 days in tissue culture, osteoblasts grown on OP-1–derived peptides 6, 11, 12, and to a lesser extent 1 and 13 were able to support BSP gene expression. In addition, peptides 6 and 11 were able to support OC gene expression. These results are consistent with the data obtained from mineralization studies. Thus, these results suggest a role for shorter BMP-7 peptides to support mineralization.

The present study has screened BMP-7 (OP-1) for bioactivity relative to in vitro mineralization induction. Numerous studies have shown OP-1 to induce bone formation. Some studies have addressed the efficacy relative to OP-1 to induce bone formation adja-

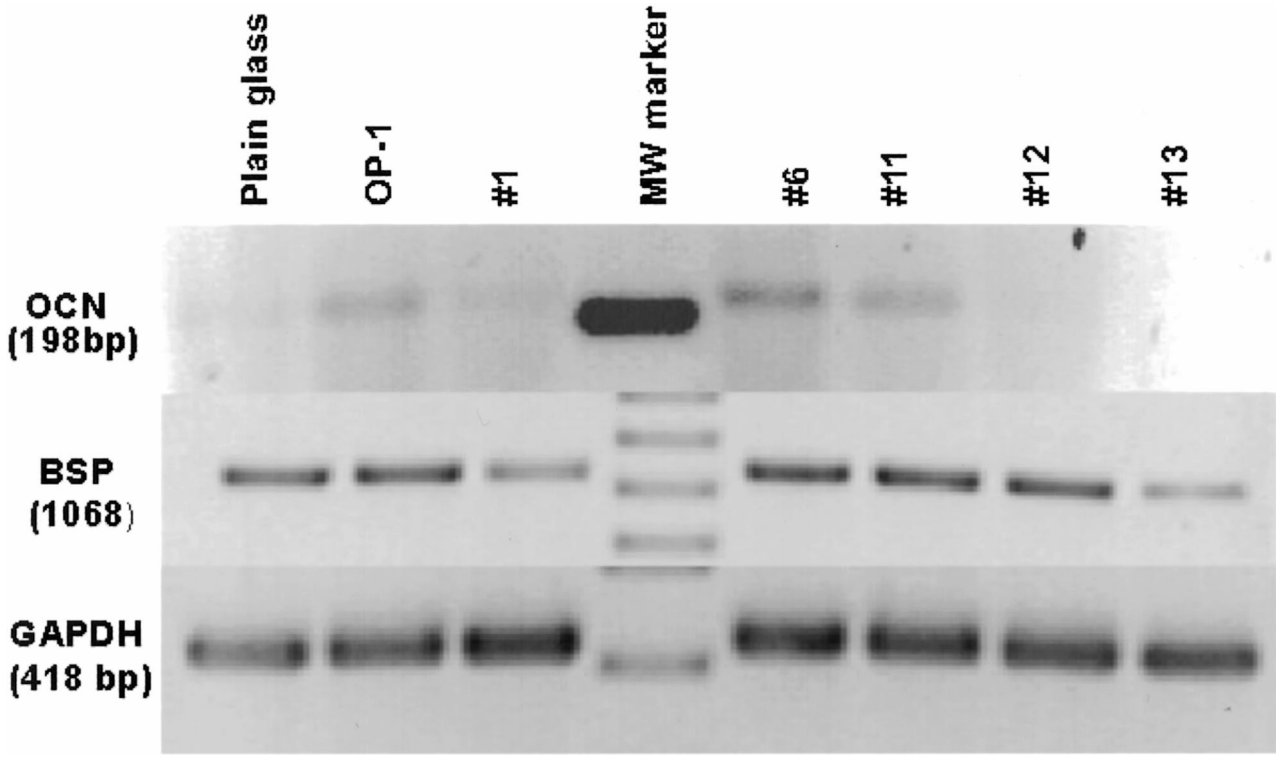

FIGURE 4. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of bone sialoprotein (BSP), osteocalcin (OC), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA expression in primary rat calvarial cells. Cells were cultured on immobilized OP-1–derived peptides for 14 days after confluency in mineralizing conditions. The RT-PCR–specific products for BSP, OC, and GAPDH were analyzed by agarose gel electrophoresis and digitized using Molecular Analyst software. Representative data from 2 independent experiments are presented.

cent to dental implants. These studies used native or recombinant OP-1. More recent studies have shown the in vitro synthesized BMP-2 containing the 102 amino acids of the C-terminal portion of the BMP-2 molecule was capable of induction of ectopic bone formation in vivo.[22] The present study represents the first attempt to synthesize peptides and explore the possibility of induction of bone formation. As shown in Figures 2 and 3, OP-1–derived peptides 1, 6, 11, 12, and 13 were able to induce or support rat calvarial osteoblastic cell mineralization and expression of noncollagenous genes consistent with the ability to synthesize bone matrix after 14 and 21 days in culture.

As a member of the TGF-β superfamily of genes, OP-1 contains 7 conserved cysteines (Cys) in the C-terminal domain that are capable of exerting its biological effect through the interaction and association of specific type I and II serine-threonine kinase receptors. Various members of the TGF-β family, including OP-1, have been shown to bind to specific type II receptors, which then complex with type I receptors to initiate signal transduction. The 3-dimensional structure of the OP-1 was determined through X-ray crystallography at 2.8-A resolution.[37] These authors proposed that the region of this molecule that contributes the ligand binding domain consists of part of finger 1 and finger 2 (Figure 5). The most intriguing finding of this article is that peptides 6, 11, 12, and 13 map the finger 1 (peptide 6) and finger 2 (peptides 11, 12, and 13) regions. The results of this study are consistent with the ability of these peptides to participate in ligand-receptor interactions and subsequent signal transduction events. In addition, peptides from this study that show promise in bone-inducing activity are mostly from the C-terminal end of the mature OP-1, similar to findings with the larger BMP-2 peptide.[22] Moreover, both C-terminal regions of BMP-2 and BMP-7 show a high degree of structural homology in this region.[38] We speculate that combinations of OP-1 peptides from the C-terminal may offer additional bone-inducing activity. Ongoing studies will address if these peptides can initiate BMP-mediated signaling processes known to be involved in BMP-7 signaling events, such as phosphorylation of SMAD-1 and SMAD-5 or MAP kinases.

The preliminary evidence from this study may provide a novel set of OP-1–derived peptides that are capable of inducing bone formation. However, additional studies are needed to substantiate these findings and to identify the molecular mechanisms by which these peptides may mediate their events.

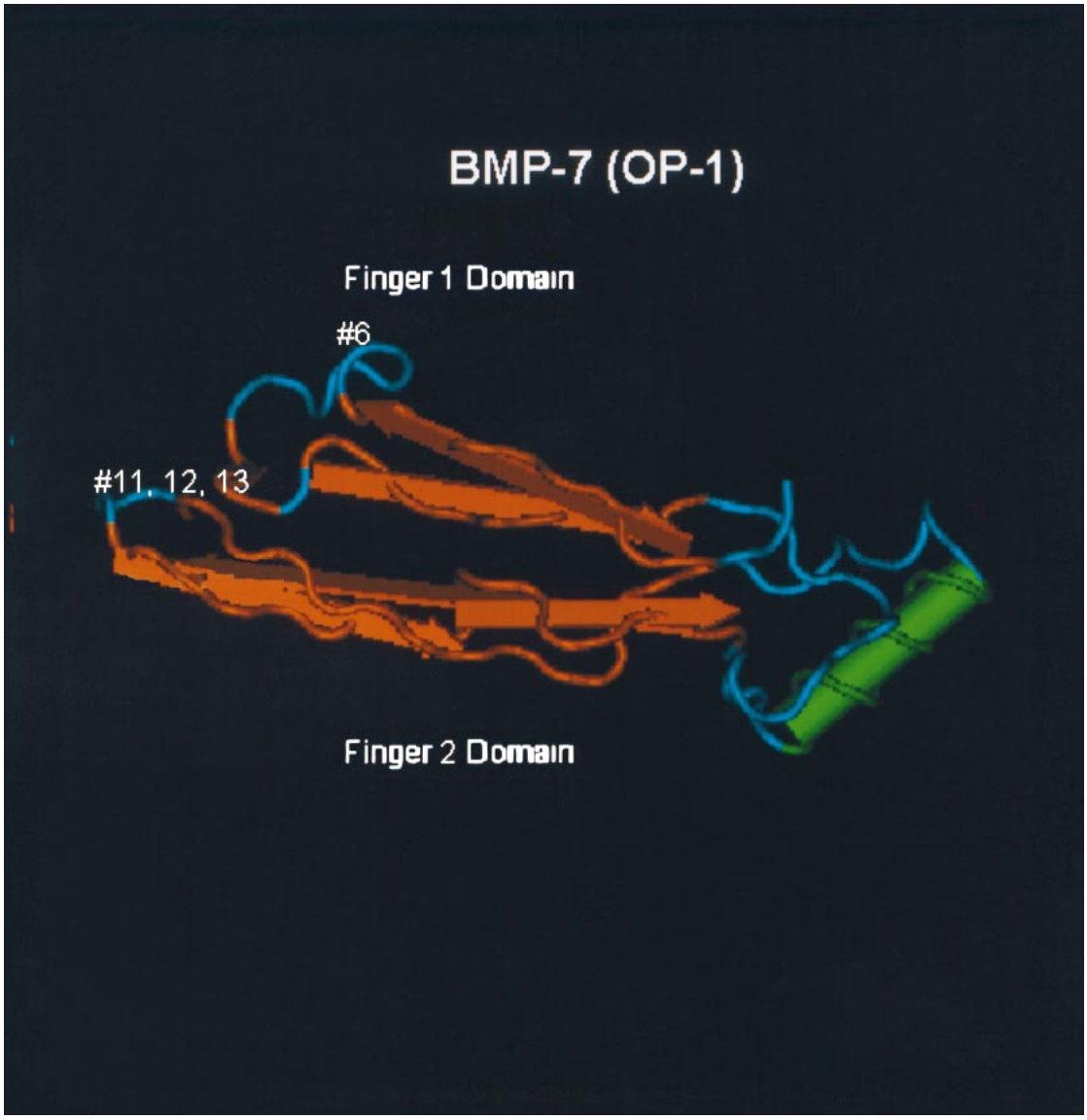

FIGURE 5. Bioactive osteogenic protein 1 (bone morphogenetic protein 7 [BMP-7]) peptides map to regions of the BMP-7 molecule involved in ligand-receptor interaction regions. Peptide 6 is located within the finger 1 domain and peptides 11, 12, and 13 comprise the finger 2 domain. Both finger 1 and finger 2 are believed to participate in ligand-receptor interaction and signal transduction (see ''Discussion'' section for details). Image was captured from the Molecular Modelling Database using the Cn3D 4.0 application program.

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