

Substrate-immobilized bone morphogenic protein-7 peptides on titanium surface support the expression of extracellular matrix proteins

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I. INTRODUCTION

Bone regeneration is an attractive area of research within tissue engineering because of the demanding clinical requirements of bone repair. It is widely recognized that various osteogenic growth factors, such as bone morphogenic protein (BMP), transforming growth factors β 1 (TGF- β 1), and basic fibroblast growth factor, regulate the proliferation and differentiation of osteogenic cells and enhance bone formation¹⁾. Thus if one can accelerate bone regeneration using osteogenic growth factors in a suitable manner, this regeneration technology will provide a new clinical procedure to promote bone repair and be a substitute for autogenous and allogeneous bone grafts or biomaterial implants.

BMPs, with their potential to promote bone formation in vivo, have been used for bone re-

generation to repair bone injuries and defects²⁾. The current DNA technologies have enable the production of enough recombinant human BMPs for basic and applied research. BMP-2 and BMP-7 have already been clinically applied to accelerate bone regeneration, both in fracture healing and spinal fusion³⁾. On the other hand, recombinant osteogenic protein 1 and partially purified osteogenic protein preparations have been shown to enhance the osseointegration around dental implants^{4,5)}. Osteoblast differentiation and mineralization have been demonstrated on titanium, glass, and hydroxypapatite-coated implant surface^{6,7)}. Using various methods, including immunocytochemical analysis and electron microscopy, no significant differences have been observed between implant surface coatings and mineralization in tissue culture. Thus, more recent experimental strat-

이 논문은 2004년도 전남대학교 특별연구사업비 지원에 의하여 연구되었음.

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Table 1. Sequence of rh BMP-7-derived peptides

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

egies have focused on recombinant protein use to enhance bone formation around titanium implants^{8,9)}.

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^{10,11)}. The use of biomimetic engineered surfaces have mainly used adhesive peptides. Relatively fewer studies have used BMP-derived peptides to determine if these peptides are osteogenic¹¹⁻¹⁴⁾. We reported the initial results that rh BMP-7 derived peptides on glass coverslips enhanced peptide-induced mineralization¹⁵⁾. However, additional studies are needed to support the initial results and to identify the molecular mechanism by which these peptides may mediate their events. Therefore, the purpose of this study was to identify whether rh BMP-7 derived peptides on glass coverslips are able to support the osteoblast differentiation via the expression of extracellular matrix proteins such as, bone sialoprotein and osteocalcin in primary rat calvarial cells.

II. MATERIALS AND METHODS

1. rh BMP-7 peptides preparations

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

The mature form of the human recombinant bone morphogenic protein 7 (rh BMP-7) osteogenic protein 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.

2. Cell culture of primary rat calvarial cells

Osteoblast-enriched cell preparations were obtained from SD rat 21-day fetal rat calvaria by sequential collagenase digestion in bone cell buffer. These cells were pooled and cultured in

BGJb media with 10% FBS at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

3. Mineralization Assay

Primary rat calvarial cells were seeded in 12-well plate containing peptide-immobilized or control glass coverslips at an initial cell density of 10,000 cells per well 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 camera and a Nikon SMZ-U stereomicroscope at x 5 magnification. Optimas image-processing software (Media Cybernetics, CA, U.S.A.) was used to detect and quantify darkly stained (mineralized) regions of the culture surface areas. One-way ANOVA was used to determine whether the culture condition affected the degree of mineralization.

4. Alkaline Phosphatase (ALP) Activity

The assay for ALP activity was carried out according to Bretauiere and Spillman¹⁶⁾. For this purpose cells were seeded onto 12 well dishes at a density of 1×10^5 cell/ml in the BGJb medium containing 10% FBS, ascorbic acid 40 µg/ml and 20 µg/ml β-glycerol phosphate. Determination of ALP activity was performed as follows; At the day 7, cells were washed with PBS, lysed in Triton 0.1% (Triton X-100) in PBS, then frozen at -20°C and thawed. 100 µl of cell lysates was mixed with 200 µl of 10 mM p-nitrophenyl phosphate and 100 µl of 1.5 M 2-amino-2-methyl-1-propanol buffer. Samples

were then incubated for 1 hour at 37 °C. ALP activity was measured for each sample by absorbance reading at 405 nm with a spectrophotometer (SmartSpec™, BioRAD, U.S.A) and corrected for cell number determined in parallel. All experiments were carried out in triplicate.

5. Total RNA extraction and RT-PCR analysis

Primary rat calvarial cells were plated into 6-well dishes containing coverslips or titanium (12mm × 12mm × 0.5 mm) with immobilized peptides or control coverslips. Total RNA from peptide-immobilized and control osteoblast cell populations was isolated using Trizol[®] (Life technology, U.S.A) at day 14. Extracted RNA was quantitated by spectrophotometer.

A total RNA of 5 µg of total RNA was used for complimentary DNA synthesis with oligo(dT)12-18 primer and Superscript II in reverse RT reaction. A total of 2 µl of RT product was used as a template for PRC amplification of bone sialoprotein (BSP), osteocalcin (OCN), and GAPDH (Table 2). 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.

6. Western blot analysis

Primary rat calvarial cells are exposed to BMP-7 derived peptides for 2 hours. Cells are rinsed with ice-cold PBS (phosphate buffered saline) and whole cell lysates prepared in SDS-PAGE buffer. Protein concentrations are

Table 2. Amplification primer sets and conditions used in polymerase chain reaction. GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase; COL I, type 1 collagen; BSP, bone sialoprotein; and OCN, osteocalcin.

PCR primers					
Primer	Expected base pairs		Sequence (5'-3')		
GAPDH-sense (+)	418		CACCATGGAGAAGGCCGGGG		
GAPDH-antisense (-)			GACGGACACATTGGGGGTAG		
COL I-sense (+)	250		TCTCCACTCTTCTAGGTTCT		
COL I-antisense (-)			TTGGGTCATTTCCACATGC		
BSP-sense (+)	1068		AACAATCCGTGCCACTCA		
BSP-antisense (-)			GGAGGGGGCTTCACTGAT		
OCN-sense (+)	198		TCTGACAAACCTTCATGTCC		
OCN-antisense (-)			AAATAGTGATAACCGTAGATGC		

PCR primers					
	94 °C	94 °C	60 °C	72 °C	72 °C
	1 min	1 min	2 min	1 min	10 min
GAPDH		<div> <div></div> <div></div> <div></div> </div>			
		25 Cycles			
	94 °C	94 °C	55 °C	72 °C	72 °C
	1 min	1 min	2 min	1 min	10 min
COL I		<div> <div></div> <div></div> <div></div> </div>			
		30 Cycles			
	94 °C	94 °C	50 °C	72 °C	72 °C
	1 min	1 min	2 min	1 min	10 min
BSP		<div> <div></div> <div></div> <div></div> </div>			
OCN		<div> <div></div> <div></div> <div></div> </div>			
		30 Cycles			

measured by Bradford's method. 10 µg of each sample is electrophoresed on 10% denatured SDS-PAGE gels, transferred to nitrocellulose membranes. Antibody against phosphorylated form of SMAD 1, 5, 8 is used as primary antibody. Primary antibody is detected using HRP-conjugated secondary antibodies and LuminoGlo

chemiluminescence detection.

7. Statistical analysis

An analysis of variance (ANOVA) for replicate measurements and DUNCAN multiple range test was done using SAS program.

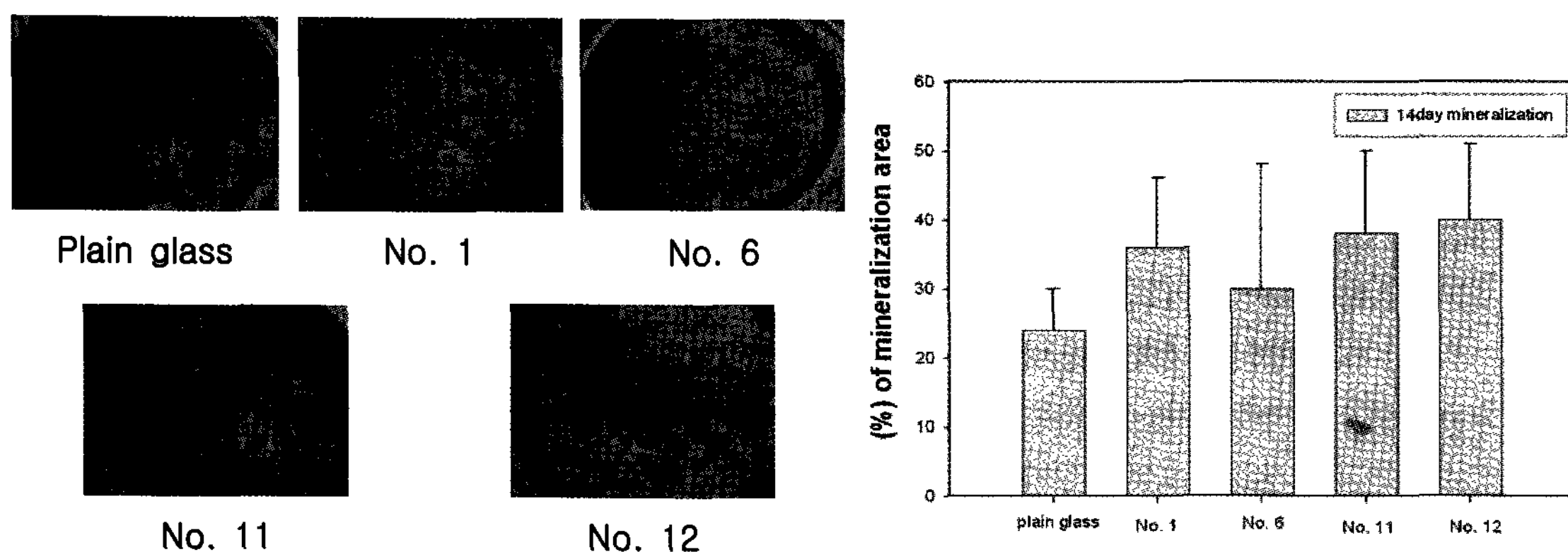


Figure 1. Representative in vitro mineralization data obtained from 14-day cultures of primary rat calvarial cells.

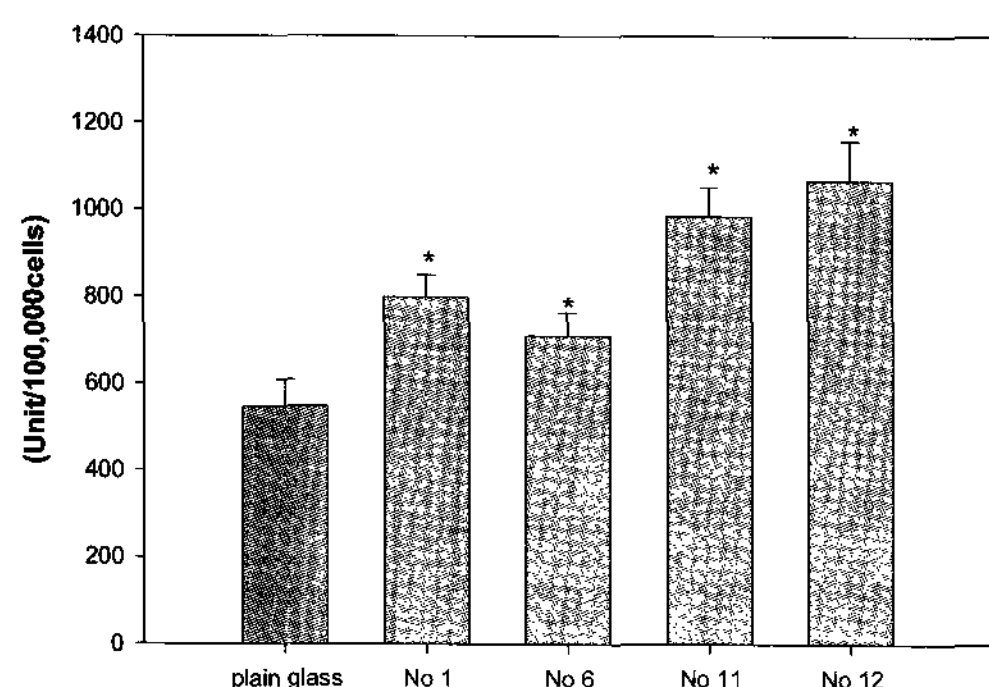


Figure 2. Alkaline phosphatase activity of rat periodontal ligament cells treated with BMP-7 (U/100,000 cells). *: indicates significantly difference with plain glass ($p < 0.01$).

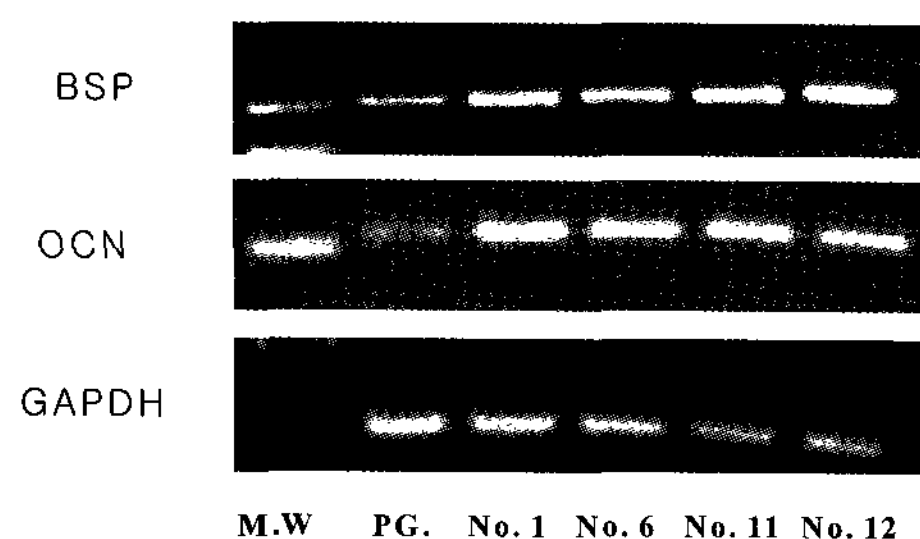


Figure 3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of bone sialoprotein (BSP), osteocalcin (OCN), and glyceral-dehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA expression in primary rat calvarial cells.

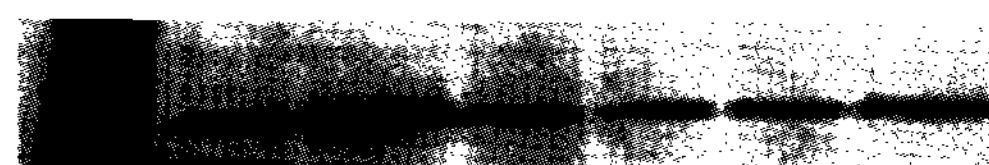


Figure 4. Western blot analysis of phosphorylated form of SMAD 1-5-8 antibody.

III. RESULTS

From initial screening, 4 candidate BMP-7 peptides (peptide No 1, 6, 11, 12) were used in this study of mineralization, alkaline phosphatase activity, and gene expression. Mineralization of

samples were shown in figure 1. In figure 2, we showed mineralization data of 14-day periods normalized to tissue culture plastics. 4 candidate BMP-7 peptides had the ability to produce mineralized nodules of rat calvarial cells at 14 days. Although variable induction on minerali-

zation occurred on 4 BMP-7-immobilized substrates at 14-day periods, the percentage of mineralized bone nodule were more than plain glass control (Figure 1).

Effects of BMP-7-immobilized substrates on ALP activity in primary rat calvarial cells are shown in figure 3. At 7 days, ALP activities of 4 BMP-7-immobilized substrates were higher than plain glass control (Figure 2, $p < 0.01$).

Using RT-PCR analysis, peptides 1, 6, 11, 12 were used to evaluate the potential of these peptides to support messenger RNA expression of osteoblastic-specific genes associated with noncollagenous bone matrix formation, namely bone sialoprotein (BSP) and osteocalcin (OCN) expression compared with controls of plain glass. In figure 3, 4 BMP-7-immobilized substrates induced BSP and OCN mRNA compared with controls of plain glass. 4 BMP-7-immobilized substrates and controls were able to supports BSP expression compared with control at 14 days (Figure 3).

In western blot analysis, neither 4 BMP-7-immobilized substrates nor controls activate phosphorylated form of Smad 1, 5, 8 (Figure 4).

IV. DISCUSSION

Improved understanding of cellular/molecular events which occur at the tissue-implant interface is contributing to a proactive approach of biomaterial design: the challenge of producing biomaterials which are engineered to elicit specific, desired responses from a patient's body^{17,18}. A biomaterial which has been engineered to enhance osteoblastic mineral deposition could, therefore, be of great clinical use.

BMPs include a large number of proteins be-

longing to the TFG- β superfamily and are characterized by their ability to induce bone and cartilage formation. Since BMPs can now be isolated and purified using recombinant technology, the effects of single BMPs can be evaluated in animal models¹⁹. The primary effect of BMP-7 on pluripotential cells that are capable of differentiating into osteoblasts appears to be its ability to commit the cells to an osteoblastic pathway^{20,21}. So, this study was designed to determine whether human BMP-7-derived peptides had the ability to support or induce biomineralization through in vitro studies. Using the linear sequence, as shown Table 1, BMP-7 was divided into 13 peptides, each of 20 amino acids in length and overlapping by 10 amino acids.

Whether primary cell or immortalized cell lines would be used when testing biomaterial is controversial. Primary osteoblasts have a diploid chromosome pattern, are characterized by growing slowly and have a finite lifespan²². Established cell lines, on the other hand, have an aneuploid chromosome pattern, tend to multiply rapidly and, if appropriately subcultured, have unlimited lifespan. An advantage of using a permanent cell line is that it provides phenotypically consistent and stable cell populations, large enough for biochemical analysis²²⁻²⁴. However, it may not always be possible to extrapolate results from osteosarcoma cell cultures because immortalization can affect cellular behavior.

Established cell lines have been used in previous studies investing cell morphology and cytotoxicity in the presence of various BMP-7-immobilized substrates. Primary cell strains derived from living tissues are necessary and

have been recommended by the International Standard Organization for specific testing to simulate the *in vivo* situation²⁴⁾.

In this study, primary osteoblasts were obtained from the fetal rat calvaria. This is an excellent source of osteoblasts because cells from young animals proliferate rapidly. Cells from the third, fourth and fifth digests were collected because these later digests provide a more pure culture, containing most cells that express an osteoblast-like phenotype²⁵⁾. The present study has used rh BMP-7-derived peptides with rat calvarial osteoblastic cells to evaluate *in vitro* mineralization. Using these cells, we evaluated the ability of rh BMP-7 once covalently attached to glass substrated to induce or support osteoblastic differentiation as measured by nodule formation and non-collagenous bone matrix gene expression.

Alkaline phosphatase (ALP) is an enzyme belonging to a group of membrane-bound glycoproteins. Although its physiological function still remains unclear, ALP may play a key role in the formation and calcification of hard tissues²⁶⁻²⁹⁾, and its expression and enzyme activity are frequently used as makers of osteoblastic cells. In this study, cells grown on 4 BMP-7-immobilized substrates showed significantly higher alkaline phosphatase levels than on plain glass ($p < 0.01$, Figure 2). Bone sialoprotein (BSP) is a 34-kDa protein that is highly sulfated, phosphorylated and glycosylated, and is expressed almost exclusively in mineralizing connective tissues^{30,31)}. Studies on the developmental expression of BSP have shown that BSP mRNA is expressed at high levels by osteoblasts at the onset of bone formation^{32,33)}, and under steady-state conditions

in vitro BSP nucleates hydroxyapatite crystal formation³⁴⁾ indicating a role for this protein in the initial mineralization of bone. Osteocalcin (OCN) is also a well-known marker highly associated with osteoblastic phenotypes, and is expressed temporally in sites of osteogenesis during developmental stages and in healing wounds. In this study, through semi-quantitative RT-PCR in primary rat calvarial cells, 4 BMP-7-immobilized substrates expressed BSP and OCN mRNA at 14 days. BSP and OCN gene expression were also upregulated in rh BMP-7-immobilized substrates, suggesting that primary rat calvarial cells were undergoing differentiation toward the osteogenic phenotype (Figure 3).

The effect of rh BMP-7 is activated by the BMP-Smad pathway³⁵⁾. Signal transduction from cell membrane to the nucleus is initiated by binding type II surface receptors. Once activated, it associated with various specific receptor-regulated Smad proteins that link the ligand receptor signals to transcription control. Thus, these proteins transduce the BMP signal at the cell surface, cytoplasm and nucleus to generate transcriptional complexes that activate specific target genes³⁶⁾. In figure 4, it was shown that 4 BMP-7-immobilized substrates nor control induced phosphorylation of Smad in primary rat calvarial cells by Western blot analysis. It implicated that 4 BMP-7-immobilized substrates did not initiate BMP mediated signaling processes known to be involved in BMP-7 signaling events.

This study demonstrated that rh BMP-7-immobilized substrates were able to induce bone formation. However, more studies are needed to identify the molecular mechanism by which these peptides may mediate their cellular events.

V. CONCLUSION

This in vitro study was an investigation of osteoblastic functions on glass substrates modified with recombinant human bone morphogenic protein-7 (rh BMP-7). Cell responses of rh BMP-7-immobilized substrates were assessed by bone nodule formation, alkaline phosphatase analysis, reverse transcription polymerase chain reaction (RT-PCR), and western blot method.

The results are as follows;

1. rh BMP-7-immobilized substrates had the ability to produce mineralized nodules of rat calvarial cells at 14 days. The percentages of mineralized bone nodule were more than plain glass control (Figure 1).
2. ALP activities of rh BMP-7-immobilized substrates were higher than plain glass control (Figure 2, $p < 0.01$).
3. rh BMP-7-immobilized substrates induced BSP and OCN mRNA compared with controls of plain glass (Figure 3).
4. In western blot analysis, neither rh BMP-7-immobilized substrates nor controls activate phosphorylated form of Smad 1, 5, 8 (Figure 4).

These results suggest that rh BMP-7-immobilized substrates could be capable of inducing bone formation. Rh BMP-7-immobilized substrates would 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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Substrate-immobilized bone morphogenic protein-7 peptides on titanium surface support the expression of extracellular matrix proteins

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이 연구는 rh BMP-7-immobilized substrates에 대한 백서 태자 두개관 세포의 반응을 석회화 결절 측정, 알칼리 효소 분석, 역전사 중합반응 및 단백질 분석등으로 평가하여 다음과 같은 결과를 얻었다.

1. 배양 14일째, 석회화 결절 형성을 측정한 결과, rh BMP-7-immobilized substrates에서 대조군과 비교하여 더 많은 석회화 결절을 형성하였다.
2. 배양 7일에 염기성 인산 분해효소 활성도는 rh BMP-7-immobilized substrates에서 대조군에 비해 효소 활성도가 유의하게 높았다.
3. 역전사 중합반응의 결과에서 BSP 와 OCN 유전자 발현은 대조군보다 더 현저하였다.
4. 단백질 분석에서 rh BMP-7-immobilized substrates와 대조군 모두 Smad 1,5,8 단백질의 인산화를 활성화시키지 못했다.

이상의 결과 rh BMP-7-immobilized substrates는 백서 태자 두개관세포가 조골세포로의 분화와 석회화를 유도하며 따라서 rh BMP-7-immobilized substrates는 임플란트 주변의 골 형성에 유용하리라 사료된다.