The Effect of Recombinant Human Bone Morphogenetic Protein-4 on the Osteoblastic Differentiation of Mouse Calvarial Cells Affected by *Porphyromonas gingivalis*

Chang-Sung Kim

The Graduate School

Yonsei University

Department of Dental Science

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Chang-Sung Kim is approved.

Thesis Supervisor: Kyoo-Sung Cho

Chong-Kwan Kim

Jung-Kiu Chai

Yun-Jung Yoo

Bong-Kyu Choi

The Graduate School

Yonsei University

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Abstract

The Effect of Recombinant Human Bone Morphogenetic Protein-4 on the Osteoblastic Differentiation of Mouse Calvarial Cells Affected by *Porphyromonas gingivalis*

Background: A number of studies have shown effective bone regeneration induced by bone morphogenetic proteins(BMPs), but it is not clear whether the presence of periodontopathic bacteria has any significant modulation effect on the bone regeneration ability of BMPs. The present study examined whether pretreatment of mouse calvarial cells with *porphyromonas gingivalis* extracts can make a difference in their osteoblastic differentiation exerted by recombinant human bone morphogenetic protein-4 (rhBMP-4).

Methods: Primary mouse calvarial osteoblastic(MCO) cells were cultured until they reached confluence. At confluence, cells were untreated or pretreated with 1 μg/ml of sonicated *P.gingivalis* extracts(SPEs) for 2 days. After washing, the cells were further incubated in the presence of rhBMP-4(0-100 ng/ml) for 3 days. At the end of the treatment, the cells were harvested and lysed for measurement of the alkaline phosphatase(ALP) activity. Total RNA was extracted and reverse transcription-polymerase chain reaction(RT-PCR) analysis for expression of ALP mRNA was conducted. The amount of prostaglandin E_2(PGE_2) secreted into the culture supernatant was determined using enzyme immuno assay.

Results: The stimulatory effect of rhBMP-4 on ALP activity was observed in both untreated MCO cells and in cells pretreated with 1 μg/ml of SPEs in a dose-dependent manner. The ALP activities were significantly reduced in the cells pretreated with SPEs at all concentrations of rhBMP-4 used in this study when compared to cells

untreated with SPEs. Similar results were obtained in the RT-PCR analysis for ALP mRNA. Cells pretreated with SPEs released significantly larger amount of PGE_2 than untreated cells, but the treatment with 100 ng/ml of rhBMP-4 had no significant effect on the amount of PGE_2 released. These results suggest that stimulatory effect of rhBMP-4 on the osteoblastic differentiation might be significantly reduced by *P. gingivalis,* possibly through the endogenous PGE_2 pathway, but rhBMP-4 still has a stimulatory effect on osteoblastic differentiation of mouse calvarial cells affected by *P. gingivalis*.

Conclusion: Our results suggest that supplemental BMPs would be beneficial for improved treatment of osseous defects, although their biologic effect might be significantly reduced by periodontopathic bacteria.

KEY WORDS

Cell differentiation; alkaline phosphatase activity; *Porphyromonas gingivalis*; bone morphogenetic proteins, recombinant; prostaglandin E_2.

The effect of recombinant human bone morphogenetic protein-4 on the osteoblastic differentiation of mouse calvarial cells affected by *Porphyromonas gingivalis*

Chang-Sung Kim, D.D.S., M.S.D.
Department of Dental Science, Graduate School, Yonsei University
(Directed by Prof. Kyoo-Sung Cho, D.D.S., M.S.D., PhD.)

I. Introduction

Bone morphogenetic proteins (BMPs) are regarded as the members of the transforming growth factor-β superfamily by characteristic features in their amino acid sequences.[1,2] A number of studies have demonstrated the biologic activities of BMPs which include the induction of ectopic cartilage and bone formation at implanted sites in vivo[2-4] and the stimulation of osteoblastic phenotype expression, such as alkaline phosphatase(ALP), type I collagen, osteocalcin, osteopontin and bone sialoprotein during the course of osteoblastic differentiation in various types of cells in vitro.[5-12] In addition, it was reported that rhBMP-2 decreases the mRNA expression of collagenase-3 by osteoblasts, whereas it increases the mRNA expression of tissue inhibitors of matrix metalloproteinases 1 and 3.[13-15] Consequently, it could be suggested that BMPs not only increase the bone matrix, but they also have the capability of preventing its degradation. However, these biologic activities of BMPs may be modulated by local environment conditions**.** Takiguchi et

al. reported that the biologic activity of rhBMP-2 on the osteoblastic differentiation in human periodontal ligament cells might be modulated by prostaglandin E_2 (PGE_2) in a biphasic manner depending on different concentrations.[16]

Porphyromonas gingivalis has been implicated as an important periodontopathic bacterium in the etiology and progression of periodontal diseases.[17] It has been reported that *P.gingivalis* may mediate periodontal destruction not only directly through their virulence factors,[18-21] but also indirectly by inducing complex host mediated inflammatory responses.[22-24] *P. gingivalis* has been shown to stimulate various kinds of cells to secrete increased levels of PGE_2, which has been implicated as an important mediator of host mediated inflammatory responses.[25-27] A number of studies have indicated that extracts of *P.gingivalis* could inhibit ALP activity, calcium and inorganic phosphate accumulation and type I collagen synthesis, whereas they decrease the gene expression of osteoblastic phenotype marker in cultures of various types of cells. These facts suggest that *P.gingivalis* not only stimulates osteoclastic activity but also inhibits osteoblastic activity.[18,28-31]

Therefore, it is possible that the above mentioned biologic activities of BMPs also could be modified by periodontopathic bacteria. The purpose of this study was to examine whether rhBMP-4 could exert a stimulatory effect on the differentiation of primary mouse calvarial osteoblstic(MCO) cells which were affected by *P. gingivalis*. For this purpose, we investigated the stimulatory effect of rhBMP-4 on the ALP activity of untreated MCO cells or cells pretreated with sonicated *P.gingivalis* extracts(SPEs). We also examined the effect of rhBMP-4 on the SPEs-induced PGE_2 production in the cells.

II. Materials and Methods

A. *P. gingivalis* culture and preparation of sonicated *P.gingivalis* extracts(SPEs)

P. gingivalis strain ATCC 33277 was cultured in brain heart infusion(BHI) broth[§] which contained 5 mg/ml of hemin and 0.5 mg/ml of Vit K at 37°C in an anaerobic chamber with an atmosphere containing 80% N_2, 10% H_2 and 10% CO_2. After 2 days of culture, the bacteria were harvested by centrifugation at 3200 x g for 20 minutes at 4°C in a centrifuge[‖] and washed 3 times with 1 x phosphate buffered saline(PBS). The purity of cultures was confirmed by phase-contrast microscopy and Gram staining. SPEs was prepared by sonication using a sonicator.[¶] The insoluble debris was removed by centrifugation at 12,000 x g for 5 minutes at 4°C. The supernatant was sterilized by filtering through a membrane filter with a pore size of 0.22 μm. The protein content of SPEs was determined using protein assay reagent kit[#] according to the manufacturer's instructions. The SPEs were stored at -70°C until used. The concentrations of SPEs used in the present study were based on our pilot cytotoxicity experiments.

B. The effects of SPEs

To investigate the effects of SPEs on osteoblastic differentiation, we tested the effects of 0.01, 0.1 and 1 μg/ml of SPEs on ALP activity in MCO cells grown for 8 days.

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§ Scharlau Chemie, Barcelona, Spain

‖ Model BR4i, Jouan, Winchester, VA

¶ Misonix Inc, NY, USA

Pierce, Rockford, IL

Another experiments were conducted by exposing MCO cells to SPEs for varying exposure periods(for 0, 2, 4, 6, 8 days) and ALP activity was measured.

C. Primary mouse calvarial osteoblastic(MCO) cells culture

MCO cell cultures were prepared from calvaria of 2 or 3-day old ICR mice using previously described procedures.[32-34] Briefly, mice were sacrificed by asphyxiation with 75% ethanol, and the calvaria were dissected and digested five times with 20-min intervals in α-minimum essential medium(α-MEM)** containing 0.2% collagenase†† and 0.1% dispase**.[35,36] Digestions 1 and 2 were discarded and cells collected from digestions 3 to 5 were pooled for the experiments. Cell number was determined by counting the cells in a hemocytometer. Cells were plated onto 35 mm culture dishes at a density of 2.5 x 10^4/ml in α-MEM containing 10% fetal bovine serum(FBS)** and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin sulfate).** Cells were incubated at 37℃ with 5% CO_2 for 6-7 days until they reached confluence. At confluence, culture medium was replaced with α-MEM containing 10% FBS, antibiotics, 50 μg/ml ascorbic acid‡‡ and 10 mM β-glycerol phosphate‡‡ with or without 1 μg/ml of SPEs. After 2 days, the culture media were removed and cell layers were washed 3 times with 1 x PBS and further incubated in the presence of rhBMP-4§§(0-100 ng/ml) for 3 days.

** Gibco, BRL, Gaithersburg, MD

†† Wako Pure Chemical Industries, Ltd., Osaka, Japan

‡‡ Sigma, Poole, UK

§§ R&D Systems Inc., Minneapolis, MN

The concentrations of rhBMP-4 used in the present study were based on our pilot experiments and previous studies performed by others.[37,38]

D. ALP activity measurement

At the end of the treatment, the culture media were removed and cell layers were washed 3 times with 1 x PBS and treated with α-MEM containing 0.2% collagenase and 0.1% dispase. After incubation for 60 minutes at 37℃, the cells were harvested by centrifugation at 8000 x g for 10 minutes at 4℃ in a centrifuge, and washed 3 times with 1 x PBS. And then the cells were lysed on the ice using 0.1% Tritron X-100‡‡ according to the manufacturer's instructions. ALP activity was measured with *p*-nitrophenyl phosphate as a substrate, using a commercial test kit‡‡ for assay of ALP activity. The amount of *p*-nitrophenol released was analyzed by measuring optical density(OD) at 405 nm using microtiterplate reader.‖‖

E. Reverse transcription-polymerase chain reaction(RT-PCR)

Total RNA was extracted using TRIZOL reagent®¶¶ according to the manufacturer's instructions. The concentrations of the RNA obtained were determined by measuring OD at 260 and 280 nm. Total RNA(1 μg) isolated from each sample was used as a template for cDNA synthesis. cDNA synthesis was performed in a thermocycler## at 42°C for 50 min using a commercial kit for reverse transcription¶¶ ; the reaction was terminated at 70°C. The cDNA was used immediately or stored at -20°C until used. The cDNA was amplified by PCR in a 50 μl reaction mixture containing 2 μl of each cDNA, 1 U of *Tag* polymerase,*** 1 x reaction buffer, 0.2 pmoles/μl of sense and antisense primers, and 0.2 mM dNTP mix. PCR was performed in a thermocycler.

PCR conditions and the sequences of the primers used are shown in table 1. After amplification, 16 μl of each PCR product was analyzed by electrophoresis on 1.3% agarose gel and the bands were visualized by ethidium bromide staining. The gels were photographed using gel documentation system††† under ultraviolet light. The quantitation of the band density was performed using a software program‡‡‡ for density analysis. All the data were normalized to glyceraldehyde-3-phosphate dehydrogenase(GAPDH) mRNA abundance. The "gene of ALP/gene of GAPDH" density ratios were calculated and represented in graphs.

F. Determination of PGE_2 production

At the end of the culture, the culture supernatants were harvested and the amount of PGE_2 secreted into the culture supernatant was determined using an enzyme immuno assay(EIA) kit§§§ for mouse PGE_2 according to the manufacturer's instructions.

‖ ‖ Dynatech lab., Chantilly, VA

¶¶ Gibco BRL, Gaithersburg, MD

Biometra®, Göttingen, Germany

*** Takara, Shiga, Japan

††† Gel Doc 1000, Bio-Rad, Hercules, CA

‡‡‡ Gel-pro® Analyzer version 4.0; Media Cybernetics, Silverspring, MD

§§§ Cayman Chemical, Ann Arbor, MI

cDNA	Primer	base pairs	annealing temperature (°C)	Thermal cycles
GAPDH [39]	S; AAGCCCATCACCATCTTCCAG As; AGGGGCCATCCACAGTCTTCT	361	57	25
ALP [40]	S; GCCCTCTCCAAGACATATA As; CCATGATCACGTCGATATCC	372	55	25

Table 1. Oligodeoxyribonucleotide Primer Sequences, Annealing Temperature and Thermal Cycles used for PCR.

S; Sense

As; Antisense

G. Statistical Analysis

Each experiment was performed at least 3 times. Means and standard deviations(SD) were calculated and all the data were presented as mean±SD. Statistical differences were determined by ANOVA and *post-hoc t*-test for multiple comparison and Mann-Whiteny U test for comparison between each SPEs untreated group and SPEs pretreated group with the same rhBMP-4 concentration. Statistical significance was determined at the $P<0.05$ level.

III. Results

A. The effects of SPEs on the ALP activity of MCO cells

Statistically significant reductions ($P<0.05$) in the ALP activity were observed, in a dose-dependent manner, in MCO cells grown for 8 days in the presence of 0.01, 0.1 and 1 μg/ml of SPEs when compared to control cells which were not treated with SPEs(Fig.1). At 0.01 μg/ml of SPEs, ALP activity was significantly reduced to 70% compared to that of control cells, and further reduced to 39% when treated with 1 μg/ml of SPEs.

We also investigated the effect of SPEs (1 μg/ml) on the ALP activity for different exposure periods. Statistically significant inhibition (65% of the control level) of ALP activity was shown at 2 days of exposure and further significant inhibition occurred by extending the periods of exposure (Fig. 2). These results indicated that SPEs have a significant inhibitory effect on the differentiation in MCO cells by relatively short exposure, as represented by reduction of ALP activity.

B. Modulation of rhBMP-4-stimulated ALP activity by pretreatment with SPEs

Fig.3 summarizes the dose-dependent effect of rhBMP-4 on ALP activity in untreated MCO cells or in cells pretreated with 1μg/ml of SPEs. As shown in Fig.3, the rhBMP-4 used in this study has a biologic activity to stimulate the ALP activity in MCO cells in a dose-dependent manner. At 10 ng/ml of rhBMP-4, ALP activity level was significantly enhanced to 262% of the cells without rhBMP-4 level, reaching 415% of the cells without rhBMP-4 level at 100 ng/ml of rhBMP-4.

In the cells pretreated with SPEs, ALP activity was also significantly enhanced to

228% of the cells without rhBMP-4 level at 10 ng/ml of rhBMP-4. Although dose-dependent stimulation of ALP activity was also observed, concentrations of more than 10 ng/ml of rhBMP-4 failed to show statistically significant increase of ALP activity. Compared to cells untreated with SPEs, the ALP activities were significantly reduced at all concentrations of rhBMP-4 used in this study.

C. Modulation of rhBMP-4-stimulated expression of ALP mRNA by pretreatment with SPEs

Fig.4 shows the dose-dependent effect of rhBMP-4 on the expression of ALP mRNA in untreated MCO cells or in cells pretreated with 1 μg/ml of SPEs. The expression of ALP mRNA was dose-dependently increased by rhBMP-4 in both untreated cells and in cells pretreated with SPEs(Fig.4A). In both groups, distinctive bands were found following the treatment with rhBMP-4 at concentrations of 50 and 100 ng/ml. However, the cells pretreated with 1 μg/ml of SPEs expressed lower level of ALP mRNA when compared to the untreated cells in the density ratio analysis (Fig.4B), reaching 50% and 57% of that of the untreated cells at concentrations of 50 and 100 ng/ml of rhBMP-4, respectively.

D. The amount of PGE_2 secreted in culture supernatant

Table 2. summarizes the effects of rhBMP-4 on the amount of PGE_2 released into culture supernatant by untreated MCO cells or cells pretreated with SPEs. The amount of PGE_2 secreted into culture supernatant was larger in MCO cells pretreated (the order of 10^{-7}M) than untreated (the order of 10^{-8}M), but the treatment with 100 ng/ml of rhBMP-4 had no significant effects on the amount of PGE_2 released.

MCO cells	rhBMP-4(ng/ml)	
	0	100
SPEs untreated(10^{-8}M)	2.23±0.27	5.79±1.11
SPEs pretreated(10^{-7}M)	1.85±0.28*	1.54±0.28*

Table 2. The effects of rhBMP-4 on the PGE_2 release by MCO cells pretreated or untreated with 1μg/ml of SPEs. The amount of PGE_2 released into the culture supernatant was determined by an EIA kit. Results are expressed. The data shown are the mean±standard deviation. The experiments were conducted at least 3 times and similar results were obtained.(*; statistically significant different(P<0.05) from SPEs untreated MCO cells)

IV. Discussion

BMPs are considered to play an important role as a regulator of the bone metabolism and have a biologic activity to induce osteoblastic differentiation in various kinds of cells. Recent studies indicate that activity of BMPs on osteoblastic differentiation could be modulated by a variety of inflammatory cytokines, such as IL-1β[12,41] and PGE_2.[16] Several studies indicated that *P. gingivalis* could stimulate various kinds of cells to secrete increased levels of PGE_2, which has been implicated as an important mediator of host mediated inflammatory responses.[25-27] Thus, it is probable that biologic activity of BMPs could be modulated by *P. gingivalis*, which has been implicated as an important periodontopathic bacterium. In case of platelet drived growth factor-BB (PDGF-BB), it has been demonstrated that lipopolysaccharides(LPS) isolated from *P. gingivalis* could modify mitogenic and chemotactic response of the periodontal ligament cells to PDGF-BB, possibly through change in PDGF-receptor protein.[42-45] However, it is not known whether the biologic activity of BMPs could be modulated by *P. gingivalis*. In the present study, we observed that the biologic activity of rhBMP-4 on osteoblastic differentiation was reduced by pretreatment with 1 μg/ml of SPEs when ALP activity and expression of ALP mRNA were analyzed. To our knowledge, the findings presented here are the first indication that the biologic activity of BMPs on osteoblastic differentiation might be modulated by periodontopathic bacteria.

As demonstrated in Fig.3, in the cells pretreated with SPEs, the ALP activity was significantly reduced at all concentrations of rhBMP-4 used in this study compared to the cells untreated with SPEs although dose-dependent stimulation of ALP activity was also observed. Moreover, similar findings were also observed in results obtained

from the RT-PCR analysis for expression of ALP mRNA(Fig. 4). Therefore, it could be suggested that *P. gingivalis* has an inhibitory effect on rhBMP-4-stimulated osteoblastic differentiation, although the precise molecular mechanism(s) responsible for reduction of rhBMP-4-induced ALP activity in MCO cells pretreated with SPEs is not fully understood. However, rhBMP-4 might also have stimulatory effect on differentiation of osteoblast affected by *P. gingivalis*.

P. gingivalis has been reported to mediate the destruction of periodontal tissue either directly through their virulence factors or indirectly through the production of inflammatory cytokines by various kinds of host cells. It was reported that extracts isolated from *P. gingivalis* might inhibit osteoblastic differentiation and the inhibitory effects on osteogenesis might be largely irreversible when extracts were added during the early differentiation stage.[28,31] In the present study, we demonstrated that SPEs suppress the ALP activity in MCO cells in a dose-dependent manner. We also observed a significant reduction of the ALP activity in MCO cells pretreated with SPEs after relatively short exposure time(day 0-2). Therefore, these findings suggest that a certain critical irreversible changes might occur in the MCO cells by pretreatment with SPEs during the early differentiation stage.

In regard to the inhibitory effect of SPEs on the rhBMP-4 stimulated osteoblastic differentiation, there is evidence which suggests the involvement of PGE_2-mediated pathway. *P. gingivalis* has been shown to stimulate various kinds of cells to secrete increased levels of PGE_2.[25-27] PGE_2 exerts a biphasic effect on osteoblastic differentiation with low concentrations of PGE_2 enhancing ALP activity and with higher concentrations of PGE_2 showing inhibition in the MC3T3-E1 cells.[46,47] Recently, the biphasic effect of PGE_2 was also demonstrated in rhBMP-2 stimulated

osteoblastic differentiation.[16] Moreover, it was reported that osteoblastic differentiation could be inhibited by extracts obtained from periodontopathic bacteria including *P. gingivalis*, but the inhibitory effect of these bacterial extracts was blocked by indomethacin, a known inhibitor of endogenous PGE_2 production.[18,30] Therefore, we investigated the effects of pretreatment with SPEs on the amount of PGE_2 released into culture supernatant by MCO cells. As shown in table 2, the amount of PGE_2 secreted into the culture supernatant was significantly increased by pretreatment with SPEs, but the treatment with 100 ng/ml of rhBMP-4 had no significant effect on the amount of SPEs-induced PGE_2 production. Together with the previous reports, these findings suggest that a certain critical irreversible change occurred in the MCO cells used in this study by pretreatment with SPEs during the early differentiation stage, possibly through the endogenous PGE_2 pathway, and the reduction of rhBMP-4-stimulated ALP activity in SPEs pretreated cells seems to be due to the increased level of PGE_2 induced by pretreatment with SPEs.

We intend to conduct further studies concerning the change in BMP receptors and signal transduction to understand the underlying mechanisms involved in the *P. gingivalis*–induced inhibition of osteoblastic differentiation in the future since the stimulatory effects of BMPs on osteoblastic differentiation could be mediated through BMP receptors and the Smad protein pathway, which is the downstream signaling molecules.

In conclusion, the findings presented in this study demonstrate that the stimulatory effect of rhBMP-4 on osteoblastic differentiation represented as ALP activity in MCO cells is significantly reduced by pretreatment with SPEs during the early differentiation stage, possibly through endogenous PGE_2 pathway. But rhBMP-4 still

has a stimulatory effect on the osteoblastic differentiation of MCO cells affected by *P. gingivalis*. Therefore, we would suggest that supplemental BMPs would be beneficial for improved treatment of osseous defects, although their biologic effect might be significantly reduced by periodontopathic bacteria.

V.Conclusion

A number of studies have shown effective bone regeneration induced by bone morphogenetic proteins(BMPs), but it is not clear whether the presence of periodontopathic bacteria has any significant modulation effect on the bone regeneration ability of BMPs. The present study examined whether pretreatment of mouse calvarial cells with *porphyromonas gingivalis* extracts can make a difference in their osteoblastic differentiation exerted by recombinant human bone morphogenetic protein-4 (rhBMP-4), and the following results were obtained

1. The stimulatory effect of rhBMP-4 on alkaline phosphatase(ALP) activity was observed in both untreated MCO cells and in cells pretreated with 1 μg/ml of SPEs in a dose-dependent manner.

2.The ALP activities were significantly reduced in the cells pretreated with SPEs at all concentrations of rhBMP-4 used in this study when compared to cells untreated with SPEs.

3.The expression of ALP mRNA was dose-dependently increased by rhBMP-4 in both untreated cells and in cells pretreated with SPEs. However, the cells pretreated with 1 μg/ml of SPEs expressed lower level of ALP mRNA when compared to the untreated cells in the density ratio analysis, reaching 50% and 57% of that of the untreated cells at concentrations of 50 and 100 ng/ml of rhBMP-4, respectively.

4.Cells pretreated with SPEs released significantly larger amount of PGE_2 than

untreated cells, but the treatment with 100 ng/ml of rhBMP-4 had no significant effect on the amount of PGE_2 released.

These results suggest that stimulatory effect of rhBMP-4 on the osteoblastic differentiation might be significantly reduced by *P. gingivalis,* possibly through the endogenous PGE_2 pathway, but rhBMP-4 still has a stimulatory effect on osteoblastic differentiation of mouse calvarial cells affected by *P. gingivalis*. Our results suggest that supplemental BMPs would be beneficial for improved treatment of osseous defects, although their biologic effect might be significantly reduced by periodontopathic bacteria.

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Figure Legends

Figure 1. Dose-dependent effect of SPEs on ALP activity in MCO cells. Confluent MCO cells were treated with SPEs(0-1 μg/ml) for 8 days, and then ALP activity was measured as described in Materials and Methods. The data shown are the mean± standard deviation. ALP activity was significantly reduced when compared to cells without SPEs at P<0.05(*). The experiments were conducted at least 3 times and similar results were obtained.

Figure 2. The effect of exposure time to SPEs(1 μg/ml) on ALP activity in MCO cells. Confluent MCO cells were treated with or without SPEs(1 μg/ml) for different time periods, and then ALP activity was measured as described in Materials and Methods. The data shown are the mean±standard deviation. ALP activity was significantly reduced when compared to cells without SPEs at P<0.05(*). The experiments were conducted at least 3 times and similar results were obtained.

Figure 3. The dose-dependent effect of rhBMP-4 on ALP activity in MCO cells pretreated or untreated with 1 μg/ml of SPEs. Confluent MCO cells were treated with or without SPEs(1 μg/ml) for 2 days, and then exposed to rhBMP-4(0-100 ng/ml) for further 3 days. ALP activity was measured as described in Materials and Methods. The data shown are the mean±standard deviation. The experiments were conducted at least 3 times and similar results were obtained. (*; statistically significantly different(P<0.05) from SPEs pretreated MCO cells †; statistically significantly different (P<0.05)from 0 ng/ml of rhBMP-4)

Figure 4. A: The dose-dependent effect of rhBMP-4 on the expression of ALP mRNA in MCO cells pretreated or untreated with 1 μg/ml of SPEs. Confluent MCO cells were treated with or without SPEs(1 μg/ml) for 2 days, and then exposed to rhBMP-4 for further 3 days. **B**: The expression of ALP mRNA was analyzed by RT-PCR. The densities of the bands were measured as described in Materials and Methods. The ALP/GAPDH density ratios were computed and then represented in graphs. All data were normalized to GAPDH mRNA. The experiments were conducted at least 3 times and similar results were obtained.

Figures

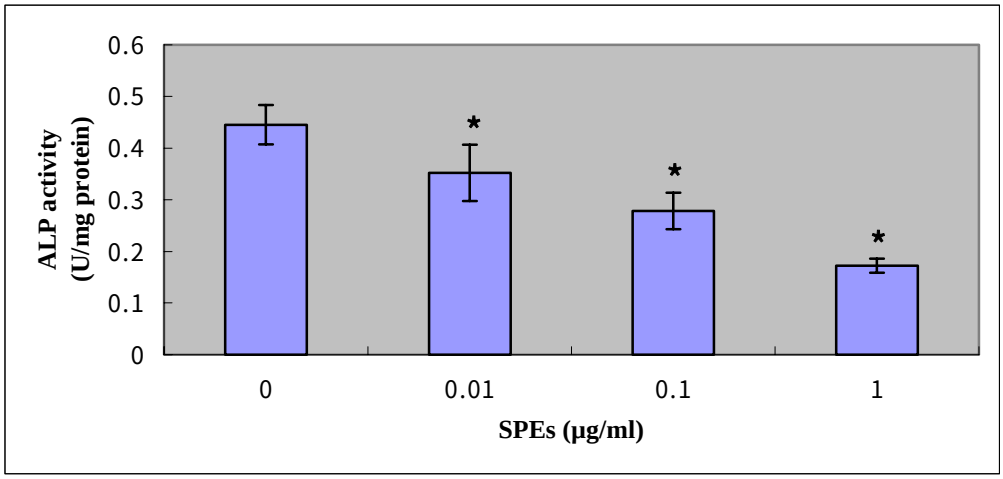


Figure 1. The dose-dependent effect of SPEs on ALP activity in MCO cells.

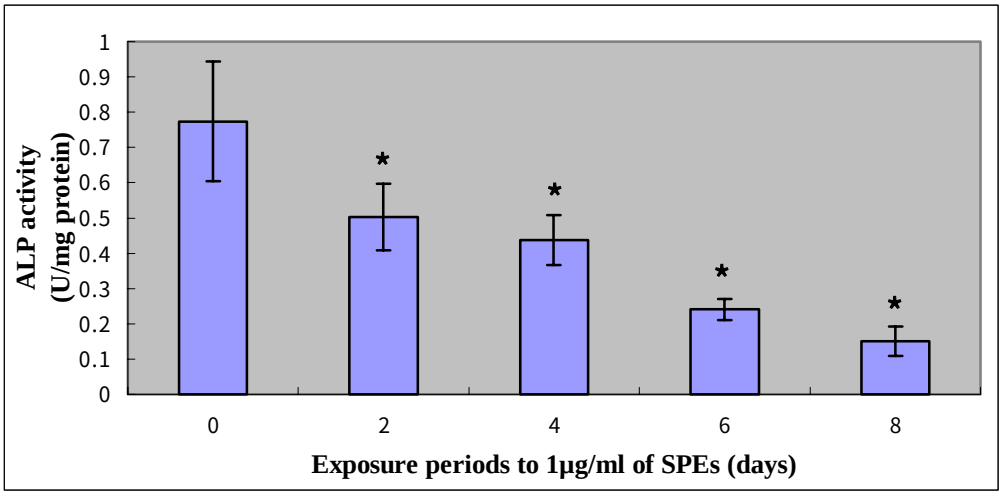


Figure 2. The effect of exposure time to SPEs(1 μg/ml) on ALP activity in MCO cells.

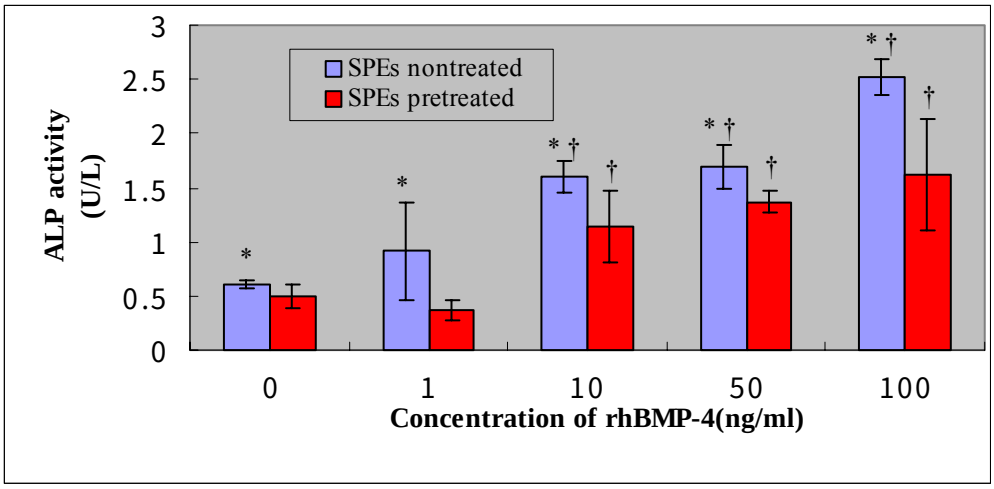
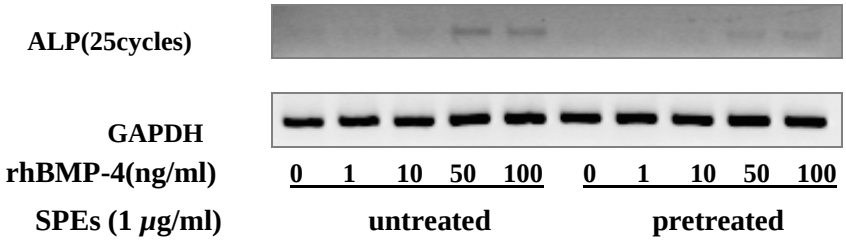


Figure 3. The dose-dependent effect of rhBMP-4 on ALP activity in MCO cells pretreated or untreated with 1 μg /ml of SPEs.

A



B

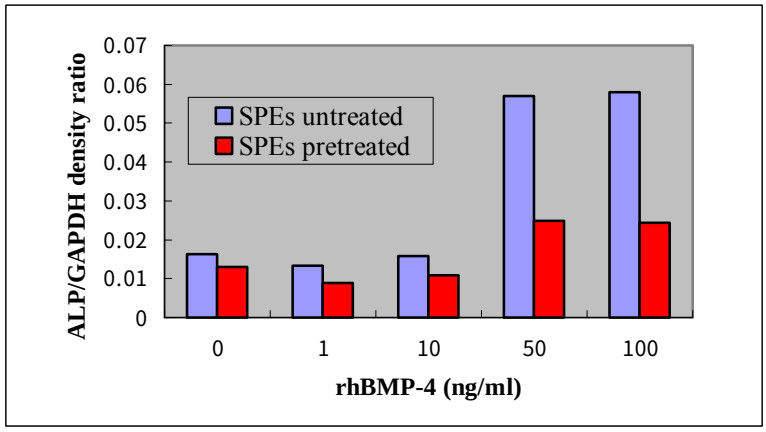


Figure 4. A: The dose-dependent effect of rhBMP-4 on the expression of ALP mRNA in MCO cells pretreated or untreated with 1 μg/ml of SPEs. **B**: The expression of ALP mRNA was analyzed by RT-PCR.

국문 요약

골 형성 유도 단백질(Bone Morphogenetic Proteins;BMP)에 의한 골 재생 유도 효과는 수많은 연구를 통하여 입증되었지만, 치주 원인 균에 의하여 골 형성 유도 단백질의 골 재생 유도 효과가 변화될 수 있는지는 명확하지 않다. 본 연구의 목적은 대표적 치주 원인 균의 하나인 *Porphyromonas gingivalis*의 분쇄 추출물로 백서태자 두개골 세포를 전 처리하였을 때 재조합 인간 골 형성 유도 단백질-4(recombinant human BMP-4;rhBMP-4)에 의한 세포의 골성 분화에 미치는 영향을 알아보고자 함이다. 백서태자의 두개골 세포를 무균적으로 분리, 배양하여 단일 층의 밀생 상태가 되면, 1 μg/ml의 분쇄 추출물로 2일간 처리하였다. 세척 후, 세포들을 rhBMP-4(0-100 ng/ml)의 처리하에 3일간 추가적으로 배양하였고, 실험 종료 후 세포들을 수집, 분쇄하여 염기성 인산 효소(alkaline phosphatase ;ALP)의 활성도를 측정하였다. RT-PCR 분석법을 이용하여 ALP mRNA 발현 정도를 측정하였고, 배양액 내로 유리된 prostaglandin E_2 (PGE_2)의 양을 측정하기 위하여 효소 면역 분석법을 시행하였다.

ALP 활성도는 분쇄 추출물의 처치에 상관없이 rhBMP-4의 농도를 증가시킴에 따라 모든 세포군에서 증가하였다. 그러나 분쇄 추출물을 처치한 세포군에서의 ALP활성도는 처치하지 않은 세포군에 비교하여 rhBMP-4의 모든 농도에서 통계학적으로 유의한 감소를 나타내었다. RT-PCR 방법에 의한 ALP mRNA발현 정도의 분석에서도 유사한 결과가 관찰되었다. 또한, 분쇄 추출물을 처치한 세포군에서 처치하지 않은 세포군에 비교하여 유의하게 많은 양의 PGE_2가 유리되었다.

이상의 결과를 볼 때, rhBMP-4는 세포의 골성 분화를 자극시키는 효과를

가지지만, 자극 효과는 *P. gingivalis*에 의하여 감소될 수 있음을 알 수 있으며 아마도 세포내 PGE_2경로 기전이 관여된다고 사료된다. 그러나, 분쇄 추출물을 처치한 세포군에서도 rhBMP-4에 의한 골성 분화 자극 효과가 농도 의존형으로 나타나므로, 치주 원인균에의해 손상된 골 결손부의 골 재생 치료에 보조적 BMP의 처치는 유익한 방법이라 할 수 있겠다.

핵심되는 말

세포 분화; 염기성 인산 효소 활성도; *Porphyromonas gingivalis*; 골 형성 유도 단백질, 재조합; prostaglandin E_2.