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Effect of Bone Morphogenetic Protein-7 on Gene Expression of Bone Morphogenetic Protein-4, Dentin Matrix Protein-1, Insulin-like Growth Factor-I and -II in Cementoblasts *In Vitro*

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ABSTRACT

Formation of root cementum is a crucial moment in the development of the periodontium. Cells that produce the cementum are named cementoblasts and they possess some unique characteristics, which differentiates them from osteoblasts. Bone morphogenetic proteins (BMPs) are crucial regulators of both bone and tooth formation. In animal studies BMPs have shown to induce periodontal regeneration, however the molecular mechanism as how BMP-7 induces cementogenesis is largely unknown. We have investigated how BMP-7 regulates gene expression of BMP-4, Dentin matrix protein-1 (DMP-1), Insulin-like growth factor-I (IGF-I) and -II (IGF-II) in cementoblasts. BMP-7 induced proliferation, and mineralized nodule formation of cementoblasts. Our results show that gene expression was influenced by the BMP-7 concentration used, with 75 ng/mL generally down-regulating gene expression at 6 hours and then up-regulating after 24 hours. The 300 ng/mL concentration had an opposite effect while the 150 ng/mL concentration generally up-regulated gene expression after 6 hours and then after 24 hours maintained this up-regulation or had no effect compared to control, depending on the examined gene. The results show that BMP-7 down-regulated BMP-4 expression in cementoblasts but still up-regulated DMP-1 gene expression suggesting that BMP-7 can, in a paracrine manner, functionally substitute for BMP-4. Furthermore, it seems that BMP-7 exerts its effect more through the IGF-II than the IGF-I pathway as shown by an up-regulation of IGF-II and down-regulation of IGF-I. These results suggest that a combination of BMP-7/IGF-II could have a potential therapeutical significance in inducing cementogenesis and periodontal regeneration.

Key words: BMP-7, cementoblasts, BMP-4, DMP-1, IGF-I, IGF-II

Introduction

It is considered that one of the crucial moments in the development of the tooth attachment apparatus is the formation of the root cementum¹. Cells that produce this thin mineralized root lining layer into which periodontal ligament Sharpey's fibers are inserted are named cementoblasts and their regulation of differentiation by growth factors is largely unknown². Although considered positional osteoblasts, cementoblasts possess some unique characteristics one of which is the relative lack of remodeling of the cementum layer. Indeed, defects in the cementum layer are associated with early-onset periodontitis³, and new cementum formation along the previously

denuded root surface is a mandatory event for a proper periodontal ligament development and periodontal regeneration⁴. Unfortunately clinical results in regenerating lost periodontal tissues are often far from 100% effectiveness and recently it has been proposed that there is a need for novel technologies to achieve a quantum leap in the application of regenerative procedures⁵.

Bone morphogenetic proteins (BMPs) have been implicated as crucial regulators of bone and tooth formation⁶⁻⁸. Since their discovery and cloning^{9,10} research has shown that BMPs are expressed in different cells during

the formation of the periodontal attachment apparatus^{11–13}. As strong candidates for their application in periodontal regeneration different BMPs act differently on periodontal regeneration. In animal studies, BMP-2 has shown a relative lack of induction of cementogenesis leading to the development of ankylosis, but induced robust alveolar bone regeneration¹⁴. Further evidence on the effect of BMP-2 on cementoblasts came from an *in vitro* study which has shown that BMP-2 inhibits mineralization and differentiation of cementoblasts¹⁵. Unlike BMP-2, animal studies investigating periodontal regeneration using BMP-7 have shown a direct effect of BMP-7 on cementogenesis by inducing complete de novo formation of new perpendicularly inserted Sharpey's fibers in to the newly formed cementum^{16,17}. Additional data on the role of BMP-7 in tooth formation came from a recent study using a conditional knockout BMP-7 mouse placing BMP-7 as a central regulator of epithelial-mesenchymal interactions that are necessary for proper tooth development¹⁸.

Another BMP that has a central role in tooth formation is BMP-4¹⁹. It has been proposed that harmonious molecular interaction between BMP-4 and noggin might be important for proper periodontium development²⁰. In BMP-4 cKO mice there is less collagen in the periodontal ligament and a complete down-regulation of dentin matrix protein-1 (DMP-1) mRNA in cementoblasts²¹, which has been shown to be important for proper cementum development since DMP-1 null mice have a defective cementum with spindle shaped cementoblasts and exhibit periodontal breakdown defects resembling early-onset periodontitis²². These results suggest that there is a role for BMP-4 and DMP-1 in cementum formation, and in addition BMP-4 has been suggested to be used in tooth bioengineering²³.

Another family of polypeptide growth factors playing a critical role in skeletal development are the insulin-like growth factors I (IGF-I) and II (IGF-II). The IGFs are the most abundant growth factors stored in bone and produced by osteoblasts²⁴. In bone IGF-II is more abundant, but IGF-I may be more potent²⁵. IGFs increase bone formation by regulating proliferation, differentiation and apoptosis of osteoblasts²⁶. The IGFs have also been linked with the development and growth of teeth and they have shown to play a role in periodontal regeneration²⁷. In human permanent teeth, using immunohistochemistry, IGF-II has been found in the acellular cementum, while IGF-I showed weak staining²⁸. Furthermore, during early stages of root resorption repair, cementoblasts within the lacunae showed IGF-II and IGF-I staining²⁹. These data indicate that the IGF system might be involved in the repair process of cementum.

The current understanding of molecular events induced by BMP-7 on cementoblasts during differentiation and mineralization of these cells is incomplete. Therefore, in the present study we examined the effect of BMP-7 on early gene expression of BMP-4, DMP-1 and IGF-I and -II in cementoblasts *in vitro*.

Materials and Methods

Cell culture

An immortalized murine cementoblast cell line (OCCM.30) (a generous gift from Dr Martha J. Somerman, University of Washington, Seattle, WA, USA) was used for these studies³⁰. OCCM.30 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) containing 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. All tissue culture reagents were from Invitrogen/Gibco BRL, Carlsbad, CA, USA.

Cell proliferation experiments

The proliferation of OCCM.30 cells was evaluated using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay. OCCM.30 cells were plated in 96-well culture plates at the density of $2,67 \times 10^4$ mL in DMEM with 10% FBS. One day after seeding, media were changed to DMEM with 5% FBS and supplemented with rhBMP-7 and rhBMP-6 (Genera Research Lab, Zagreb, Croatia) at the doses of 75, 150 and 300 ng/mL. Untreated cells served as controls. The MTT assay was performed after 24 and 48 hours. Briefly, 20 µL of MTT (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA.) was added to each well, which was converted into an insoluble blue formazan product by mitochondria of living cells but not of dying cells or debris. After incubation at 37 °C for 4 h, the supernatant was removed and the formazan crystals were dissolved in 150 µL of DMSO. Attenuance values for each well were measured spectrophotometrically at 490 nm and the assay was repeated three times.

Mineralization assay and morphological changes

Cells were plated at the density of 3×10^4 mL in 6-well culture plates and cultured in media containing 10% FBS. Upon confluence culture conditions were changed to a mineralizing media containing DMEM with 5% FBS, 50 µg/mL ascorbic acid (AA), 10 mM β-glycerophosphate (BGP) and supplemented with rhBMP-7 (Genera Research Lab, Zagreb, Croatia) at the doses of 75, 150 and 300 ng/mL. Cell mediated mineral nodule formation was assessed on day 8 with Alizarin red-S staining. A representative experiment is shown. Experiments were performed three times with comparable results.

Morphological changes of cementoblasts after 8 days in culture were observed and photographed under a phase-contrast inverted microscope (Olympus Optical, Tokyo, Japan) to evaluate their phenotype.

Gene expression analysis

– Real time Quantitative PCR

Following treatment with rhBMP-7 total RNA was isolated from OCCM.30 cells at 6 and 24 hours using TRIzol (Invitrogen, Carlsbad, CA, USA). cDNA was syn-

TABLE 1
SEQUENCES OF PRIMERS USED FOR GENE EXPRESSION ANALYSIS

| Target gene | Name | forward 5'-3' | reverse 5'-3' |
|-------------|--------------------------------|------------------------|--------------------------|
| BMP-4 | Bone morphogenetic protein – 4 | GACTTCGAGGCGGACACTTCTA | GCCGGTAAAGATCCCCTCATGTAA |
| DMP-1 | Dentin matrix protein – 1 | CAGTGAGAGCAGGGAACCTTGA | CCCTCATCGTCCAACCTCGG |
| IGF-I | Insulin-like growth factor I | GTGCTGCATCGCTGCTTAC | GGACGGGGACTTCTGAGGTCA |
| IGF-II | Insulin-like growth factor II | GTGAAGGGGAATCAGGTTACG | ACGTCCCTCTCGACTTGG |
| 18S | 18s RNA | TTGACGGAAGGGCACCACCAG | GCACCACCACCACGGAATCG |

thesized and amplified from 1 µg of total RNA using Super Script III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA.) as indicated by the manufacturer. Reactions were performed in a GeneAmp 4800 thermal cycler (PerkinElmer Life Sciences, Waltham, MA, USA). Expression of examined genes was measured using a LightCycler FastStart DNA Master SYBR Green kit in a LightCycler instrument (Roche Diagnostics, Basel, Switzerland). The expression of two housekeeping genes was analyzed and geNorm software was used to identify the most suitable reference gene. 18S RNA transcripts were used as a normalizer. The comparative CT method ($\Delta\Delta CT$) was used for relative quantification of gene expression³¹. Results are represented as a fold change of the comparative expression level. The list of primers used for BMP-4, DMP-1, IGF-I, IGF-II and 18S are shown in Table 1. Experiments were run three times with comparable results.

Statistics

All experiments in this study were performed three times, and representative findings are shown. Experimental values are given as means \pm standard error of means (SEM). One way analysis of variance (ANOVA) was performed to determine the significance between control and treatments on cementoblasts. The results were considered significant when P was <0.05.

Results

Effect of BMP-7 on cell proliferation

The effect of BMP-7 on cell proliferation was assessed. The MTT assay was conducted after 24 and 48 hours. Untreated control values are shown as 100%. After 24 hours all rhBMP-7 concentrations yielded increased proliferation with 150 ng/mL and 300 ng/mL being statistically significant ($*p < 0.05$). After 48 hours the trend of increased proliferation was still there but was not anymore statistically significant. In the rhBMP-6 treated cells after 24 hours only 75 ng/mL increased cell proliferation ($*p < 0.05$) while 150 and 300 ng/mL decreased cell proliferation. The same trend was observed after 48 hours with 300 ng/mL being statistically significant ($*p < 0.05$) (Figure 1). Subsequently for all other experiments only BMP-7 was used.

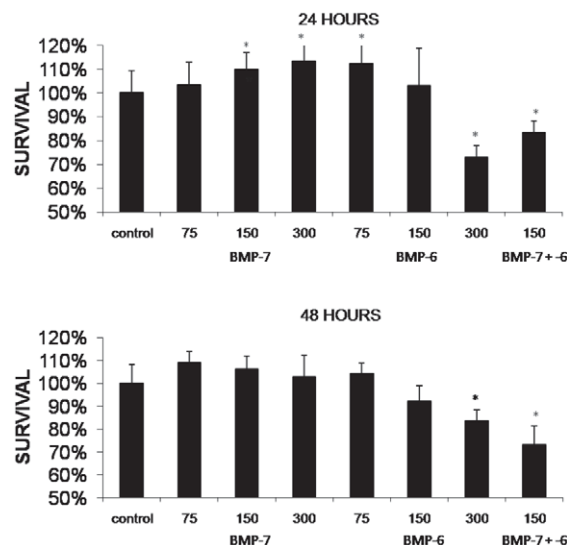


Fig. 1. Effect of different rhBMP-7 and rhBMP-6 concentrations on cementoblast proliferation. MTT assay was used to measure proliferation. Results are representative of three independent experiments; $*p < 0.05$ vs. control.

Effect of BMP-7 on cementoblasts mineralization in vitro and morphological changes

BMP-7 induced mineral nodule formation as performed by Alizarin red-S staining (Figure 2). Cells maintained only in 5% FBS did not exhibit mineral nodule formation after 8 days. The addition of mineralizing media (50 µg/mL ascorbic acid (AA) + 10 mM β -glycerophosphate (BGP)) induced mineralization at day 8, while the addition of rhBMP-7 even further induced mineral nodule formation. Similar Alizarin red-S staining was observed in the 75 and 300 ng/mL treated cells (data not shown).

Observed under a phase-contrast microscope cells treated with 150 ng/mL of BMP-7 show mineral nodule formation (Figure 3a) after 8 days and appear cuboidal and polygonal in shape (Figure 3b). Identical findings were found in the 75 and 300 ng/mL treated cells (data not shown).

Effect of rhBMP-7 on gene expression

Cells at confluence were treated with rhBMP-7 at concentrations of 75, 150 and 300 ng/mL. Total RNA was ex-

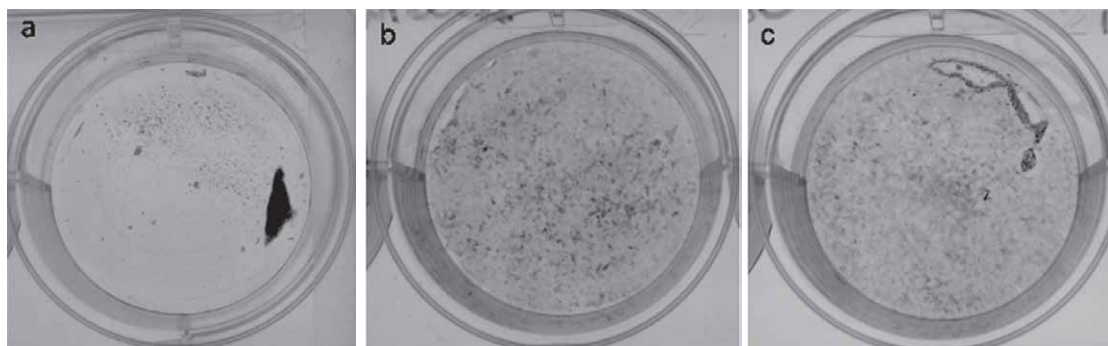


Fig. 2. Effect of BMP-7 on the mineralization behavior of OCCM-30 cells after 8 days in culture. To evaluate mineral nodule formation we performed Alizarin red-S staining. a) 5% FBS, b) 5% FBS + AA (50 µg/mL) + β-GP (10 mM), c) 5% FBS + AA (50 µg/mL) + β-GP (10 mM) + BMP-7 (150 ng/mL). Note mineralization of cells indicated by Alizarin red-S staining. Representative results of three experiments are shown here.

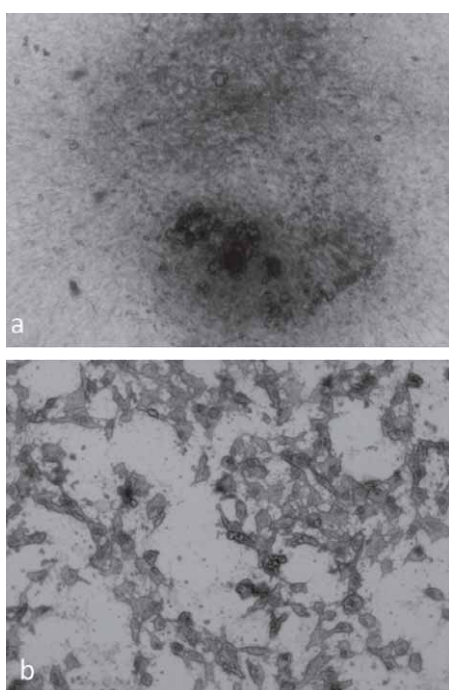


Fig. 3. Effect of BMP-7 on morphological changes of OCCM-30 cells after 8 days in culture. a) Mineralized nodule in the 150 ng/mL BMP-7 treated cells prior to Alizarin red-S staining (magnification 20x), b) Cementoblasts prior to Alizarin red-S staining showing cuboidal or polygonal shape in the 150 ng/mL BMP-7 treated cells (magnification 20x).

tracted after 6 and 24 hours to evaluate the levels of BMP-4, DMP-1, IGF-I and -II by quantitative PCR (Figure 4). The results are compared to untreated control. BMP treatment affected the expression of analyzed genes associated with mineral regulation. These genes were regulated in a dose-response fashion.

BMP-7 at a concentration of 75 ng/mL down-regulated gene expression of BMP-4 at 6 hours ($p < 0.05$), and then after 24 hours up-regulated gene expression slightly above the control level. The concentration of 150 ng/mL up-regulated (2-fold) gene expression after 6 hours ($p <$

0.05) and then down-regulated at 24 hours to slightly below the control values. The 300 ng/mL concentration down-regulated gene expression of BMP-4 at 6 and 24 hours ($p < 0.05$ / Figure 4a).

The effect of BMP-7 on DMP-1 expression shows at a concentration of 75 ng/mL a down-regulation of gene expression after 6 hours and then a statistically significant up-regulation after 24 hours ($p < 0.05$ / Figure 4b). The 150 ng/mL concentration slightly down-regulated gene expression of DMP-1 after 6 and 24 hours, while the 300 ng/mL concentration up-regulated gene expression after 6 hours and 24 hours but did not reach statistical significance.

The effect of BMP-7 on gene expression IGF-I shows that BMP-7 in all three concentrations down-regulated gene expression of IGF-I after 6 and 24 hours, but this did not reach statistical significance (Figure 4c).

BMP-7 at a concentration of 75 ng/mL down-regulated gene expression of IGF-II after 6 hours ($p < 0.05$ / Figure 4d) and then up-regulated gene expression after 24 hours. The 150 ng/mL concentration up-regulated gene expression of IGF-II at 6 (1.8-fold) and 24 (1.3-fold) hours reaching statistical significance for both ($p < 0.05$ / Figure 4d). The 300 ng/mL of BMP-7 up-regulated gene expression after 6 hours ($p < 0.05$ / Figure 4d) and then down-regulated after 24 hours below the control value but did not reach statistical significance.

Discussion and Conclusions

BMPs have been involved in the development, regeneration, and treatment of various tissues and conditions^{32–35}. Due to their presence in various cells during the formation of the periodontium^{11–13} and their ability to regenerate lost periodontal tissues¹⁴ BMPs are likely candidates for successful treatment of periodontal defects in humans. However, the molecular mechanisms as how BMPs could act on cementoblasts to induce cementogenesis are largely unknown^{15,36,37}.

BMP-4 has been associated with postnatal tooth cyto-differentiation. In BMP-4 knockout mice BMP-4 expres-

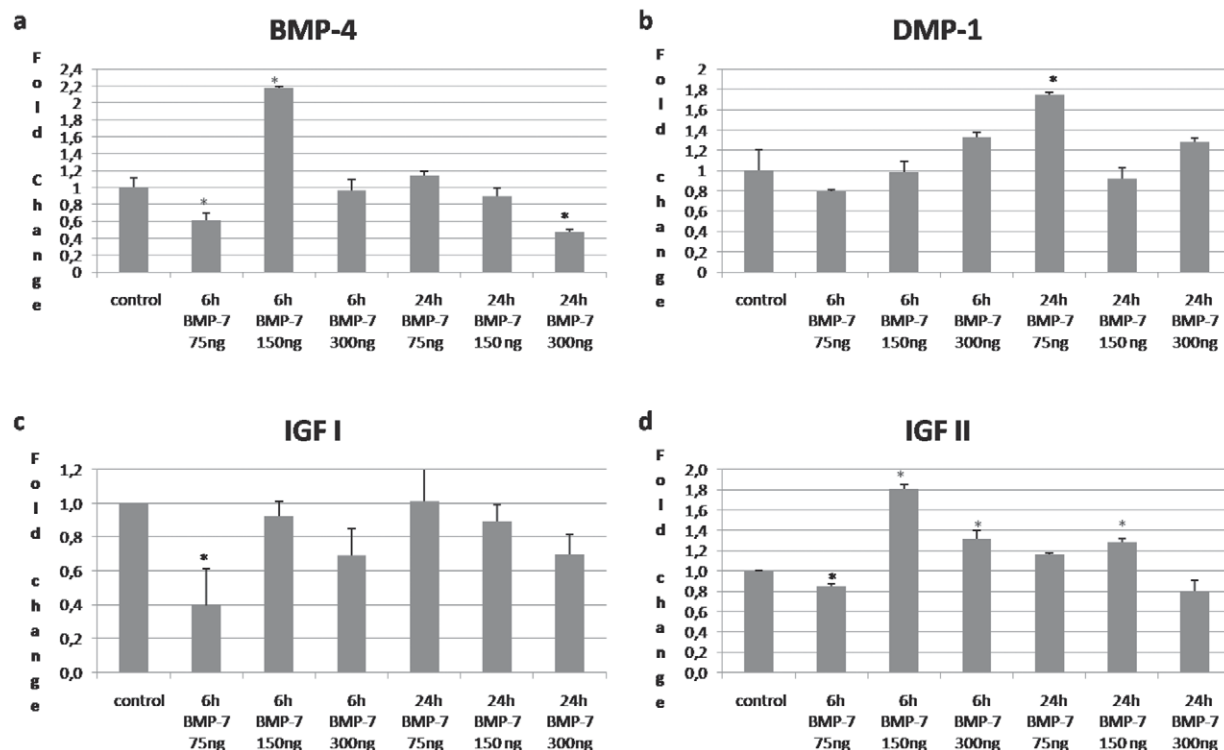


Fig. 4. Real time quantitative RT-PCR analysis of genes expressed in cementoblasts treated with rhBMP-7. OCCM.30 cells were treated for 6 (6h) and 24 hours (24h) with rhBMP-7 at concentrations of 75, 150 and 300 ng/mL. Genes analyzed include: (a) Bone morphogenetic protein-4 (BMP-4), (b) Dentin matrix protein-1 (DMP-1), (c) Insulin-like growth factor-I (IGF-I), and (d) Insulin-like growth factor-II (IGF-II). Results were normalized to 18S RNA. Results are presented as fold change compared to untreated control. Results are representative of the results obtained in three independent experiments; * $p < 0.05$ vs. control.

sion is reduced over 90% in alveolar osteoblasts and odontoblasts leading to a 97% reduction of mRNA expression of DMP-1 in cementoblasts²¹. As DMP-1 null mice exhibit periodontal breakdown and a defective cementum with an abnormal lacuna-canalliculi system²² it could be suggested that both proteins are required for proper cementum development. Furthermore, it is speculated that DMP-1 is the effective molecule in dentin non-collagenous proteins that drives dental follicle cells into cementoblasts lineages³⁸. In osteoblasts endogenous BMP-4 is required for their differentiation while exogenously added BMP-7 gradually decreased BMP-4 expression³⁹. Furthermore, in primary cultures of osteoblasts BMP-7 also suppressed BMP-4 mRNA expression⁴⁰. In our study BMP-7 after 24 hours down regulated BMP-4 expression in cementoblasts. We have shown that all three concentrations of BMP-7 can induce mineralization of cementoblasts suggesting that although there is a down regulation of BMP-4 after 24 hours, BMP-7 could in a similar manner as in osteoblasts³⁹ act as a paracrine factor in cementoblasts. It could be expected that by down-regulating BMP-4 expression in cementoblasts DMP-1 expression would also be down-regulated. Contrary to that there was an up-regulation of DMP-1 gene expression in cementoblasts following BMP-7 treatment suggesting that BMP-7 could act as a substitute in a similar manner as seen in osteoblasts. As DMP-1 null mice

have altered cementum formation rate²² by maintaining or increasing DMP-1 expression in cementoblasts BMP-7 could maintain proper cementum formation and mineralization.

Insulin-like growth factors-I and -II and their binding proteins are localized in the cementum of human teeth²⁸. Application of IGF-I/platelet-derived growth factor has shown to result in a significant promotion in bone regeneration in patients with periodontal disease⁴¹. BMPs have been shown to exert their osteoinductive effects through the activation of the IGF pathways^{42,43}. When combined, BMP-7/IGF-I synergistically stimulate osteoblastic cell differentiation⁴⁴, and in fetal rat calvarial cells BMP-7 increases pre-mRNA levels of IGF-II⁴⁵. In our study, gene expression of IGF-I and -II following BMP-7 treatment revealed that there was a down-regulation of IGF-I expression and an up-regulation of IGF-II suggesting that BMP-7 may exert its differentiation effect on cementoblasts through the activation of the IGF-II pathway. Additionally, using liquid chromatography-tandem mass spectrometry for proteomic analysis we have identified in BMP treated cementoblasts the insulin-like growth factor 2 receptor (IGF2R)/Cation-independent mannose-6-phosphate receptor, and insulin-like growth factor 2 mRNA binding proteins 1, 2 and 3 (data not shown). All of this indicates that the IGF-II pathway may play a more important role in mineralization and differ-

entiation of cementoblasts. Further support of a more important role of IGF-II than IGF-I came from a study where IGF-II gene expression was up-regulated and IGF-I down-regulated during mineralized nodule formation by periodontal ligament cells⁴⁶. The up-regulation of IGF-II, activation of its receptor and binding proteins could have potentially important clinical repercussions on periodontal regeneration and suggest that combining BMP-7/IGF-II could be a new treatment modality for periodontal tissue regeneration.

To our knowledge this is the first study to investigate the role of BMP-7 on gene expression of BMP-4, DMP-1 and IGF-I and -II in cementoblasts. In cementoblasts

BMP-7 down-regulated BMP-4 gene expression and BMP-7 treatment up-regulated DMP-1 expression. The finding that BMP-7 may exert its effect on cementoblasts through the IGF-II pathway suggests that there is valid reason to combine BMP-7/IGF-II for periodontal regeneration and the induction of cementogenesis. Future studies are needed to confirm this notion.

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IN VITRO UČINAK KOŠTANOG MORFOGENETSKOG PROTEINA-7 NA GENSKU EKSPRESIJU KOŠTANOG MORFOGENETSKOG PROTEINA-4, PROTEINA DENTINSKOG MATRIKSA-1, INZULINU SLIČNOG ČIMBENIKA RASTA-I i -II U CEMENTOBLASTIMA

S A Ž E T A K

Stvaranje cementa korijena ključan je moment u razvoju parodonta. Stanice koje proizvode cement nazivaju se cementoblastima i posjeduju neke jedinstvene osobine koje ih razlikuju od osteoblasta. Koštani morfogenetski proteini (BMP) važni su regulatori stvaranja kosti i zuba. Istraživanja na životinjama pokazala su da BMP potiču regeneraciju parodonta. Ipak molekularni mehanizmi kako BMP-7 potiče cementogenezu u potpunosti su nepoznati. Ispitali smo kako BMP-7 regulira gensku ekspresiju BMP-4, DMP-1, IGF-I i -II u cementoblastima. BMP-7 potiče proliferaciju te stvaranje mineraliziranih čvorića u cementoblastima. Rezultati pokazuju da je genska ekspresija bila ovisna o koncentraciji BMP-7 gdje je općenito 75 ng/mL smanjilo ekspresiju nakon 6 sati, a onda povećalo nakon 24 sata. Koncentraciju od 300 ng/mL imala je suprotan učinak, dok je koncentracija od 150 ng/mL nakon 6 sati povećala ekspresiju gena te ju nakon 24 sata zadržala povećanom ili nije imala učinka u usporedbi s kontrolom ovisno o kojem se genu radilo. Rezultati pokazuju da BMP-7 smanjuje ekspresiju BMP-4 ali povećava ekspresiju DMP-1 sugerirajući da BMP-7 može funkcionalno nadomjestiti BMP-4. Nadalje, čini se da BMP-7 djeluje više preko IGF-II nego IGF-I puta tako što povisuje ekspresiju IGF-II a smanjuje ekspresiju IGF-I. Ovi rezultati sugeriraju da kombinacija BMP-7/IGF-II može imati potencijalnu terapijsku važnost u poticanju cementogeneze i regeneraciji parodonta.