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BMP Expression by Human Cementum-Derived Cells *in vitro*

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Bone morphogenetic proteins (BMPs), members of a large group of TGF-beta family, are important molecular regulators of morphogenesis of numerous tissues and organs, including bones and teeth. Most BMPs are capable of inducing bone formation *in vivo* and therefore are of considerable clinical interest for regenerating mineralized tissues. Recently, we have developed a method to culture cells from human cementum (human cementum-derived cells, HCDCs). HCDCs, when attached to synthetic hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic and transplanted into immunodeficient mice, formed histologically identifiable cementum-like tissue. Since it is unclear to what extent BMPs are involved in cementogenesis, the aim of this study was to establish which BMPs are expressed by cementogenic HCDCs and whether the expression of BMPs is related to the degree of cellular differentiation *in vitro*. HCDCs were maintained in growth medium (DMEM/F12 supplemented with 10% FBS) until confluent (proliferation stage). Upon reaching confluence, cells were incubated in the differentiation medium (DMEM/F12 medium containing 10% FBS and 50 mg/ml ascorbic acid) for 14 days (differentiation stage). Next, HCDCs were incubated in mineralization medium (DMEM/F12, 50 mg/ml ascorbic acid, 2.5 mg/ml of ITS (insulin-transferrin-selenium), 5 mM beta-glycerophosphate and 10^{-8} M dexamethasone) for another 14 days (mineralization stage). At the end of each differentiation stage, total RNA was isolated and evaluated for BMPs (2 through 8) expression by employing real time RT-PCR. HCDCs expressed most of BMPs examined except BMP-7 and BMP-8. Furthermore, on average, the highest levels of BMPs were expressed at the earlier differentiation stage, prior to the initiation of mineralization *in vitro*. These results indicate that several BMPs are expressed during cementoblastic differentiation and suggest that BMPs may be involved in the homeostasis of human cementum.

Keywords : bone morphogenetic proteins (BMPs), human cementum-derived cells (HCDCs), real time RT-PCR

Introduction

Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to a large group of TGF-β superfamily that includes TGF-β, activins and inhibins (Kingsley, 1994). BMPs were identified as molecules that induce bone and cartilage formation when implanted at ectopic sites in rats (Urist, 1965, Wozney *et al.*, 1988, Reddi, 1997). Growth and differentiation of chondroblast and osteoblast lineage cells *in vitro* also are regulated by BMPs. And these molecules are important molecular regulators of growth, differentiation, chemotaxis, and apoptosis as well as morphogenesis of numerous tissues and organs (Thesleff, 1995, Hogan, 1996, Thomadakis *et al.*, 1999, Yamashiro *et al.*, 2003). According to previous BMP studies in dental field, BMPs were detected in dental tissue, including cementum, during mouse development and craniofacial abnormalities in BMP deficiencies were reported (Aberg *et al.*, 1997, Lu *et al.*, 2000, Dudas *et al.*, 2004). Taken these features together, BMPs can be regarded as potent local factors that might be used for regenerating mineralized tissue.

Cementum is mineralized tissue of the tooth and it attaches the tooth to the periodontal ligament (PDL). Cementum is similar to bone in several aspects including the fact both of them are mineralized tissues and have similar organic matrix composition. But cementum differs from bone in many aspects. Mature bones (both cortical and trabecular) in humans have a lamellar organization, whereas cementum does not, but rather

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resembles a primitive, fetal-type woven bone (Yamamoto and Hinrichsen, 1993). Bone, with few exceptions, is accompanied by bone marrow. No bone marrow is present within or around cementum. In addition, cementum is avascular and does not undergo any remodeling in normal conditions, whereas bone contains extensive vasculature and is constantly remodeled throughout life (Grant *et al.*, 1988, Freeman, 1994). However, it is still unclear whether these distinctive features of cementum are caused by its specific location and the influence of neighboring tissues, most notably PDL and dentin (Alatli *et al.*, 1996), or whether they are intrinsic to the cementoblastic phenotype.

Despite of the critical role of cementum in maintaining the structure of the periodontium and the high prevalence of periodontal disease, studying human cementum until now was limited because of the topography around cementum, contaminated by PDL cells in *in vitro* model and small number of cells, *et cetra.*

Recently, we have developed a method to culture cells from human cementum (human cementum-derived cells, HCDCs) (Grzesik *et al.*, 1998). HCDCs, when attached to synthetic hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic and transplanted into immunodeficient mice, formed cementum-like tissue. We have shown that these cells maintain the potential to form cementum-like tissue after *ex vivo* expansion and it is highly reproducible both qualitatively and quantitatively. Therefore, HCDCs represent a simple but faithful model of human cementoblastic cells indicating this can be a good tool for investigating issues related to human cementogenesis both *in vivo* and *in vitro*.

Since it is unclear to what extent locally produced BMPs are involved in the cementogenesis in a developed tooth, the aim of this study was to establish which BMPs are expressed and secreted by cementogenic HCDCs *in vitro*, to determine the pattern of BMPs expression during *in vitro* differentiation and to correlate the *in vitro* expression of BMPs by HCDCs with the capacity to form cementum in the *in vivo* assay.

Materials and Methods

Cell Culture

Healthy human premolar teeth (from patients aged 12-14 years) extracted for orthodontic reasons were used. The "human subjects" protocol was approved by the Committee on Investigations Involving Human Subjects, School of Dental Medicine, University of Pennsylvania. Teeth were either kept in serum-free Dulbeccos modified Eagle medium (DMEM)/F12 medium (Gibco-BRL, Life Technologies, Grand Island, NY, U.S.A.) containing 100 U/ml of penicillin and 100 μg/ml of streptomycin (Gibco-BRL) overnight at 4°C or processed immediately after extraction.

The cultures of HCDCs and control PDL cells were established as described (Grzesik *et al.*, 1998). Briefly, PDL was manually dissected from the tooth root with a surgical scalpel and used to establish cultures of PDL cells. After extensive washing with plain medium, teeth were subjected to collagenase P (100 mU/ml; Boehringher-Mannheim, Mannheim, Germany) treatment for 1.5-2 h at 37°C. The medium with released cells was discarded, and cementum, together with a thin layer of underlying dentin, was dissected and collected. These fragments were thoroughly washed, then minced with scissors until small chips (<0.5 mm in diameter) were obtained. The chips were next digested again with collagenase P for 1.5 h at 37°C to remove all cells unprotected by mineral. After this step, the fragments were washed thoroughly with medium and placed in 150-mm tissue culture plastic Petri dishes (Costar, Cambridge, MA, U.S.A.) containing growth medium (DMEM/F12 supplemented with penicillin/streptomycin and 10% fetal bovine serum [FBS; Gibco-BRL]). The cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Medium was changed every other day.

Under the conditions used (i.e., plating chips derived from one tooth into one 150-mm plate), HCDCs form colonies that are separated by a large distance. When the cell number within a colony reached 400-500 (usually after approximately 4 weeks in culture), cells from individual colonies were scraped with a Pasteur pipette, transferred to the wells of 12-well tissue culture plates (Costar), and cultured in the growth medium until semiconfluent (single colony-derived strains [SCDSs]). Cells that remained on the original plate were further incubated until semiconfluent (usually up to 6-7 weeks) and passaged, thus providing multicolony-derived strains (MCDSs).

6 strains of HCDCs (2MCDSs and 4 SCDSs) were used in this study.

In vitro Differentiation

To establish *in vitro* differentiation, cells were maintained in growth medium until confluent (proliferation stage). Upon reaching confluence, cells were incubated in the differentiation medium (growth medium supplemented with 50 mg/ml ascorbic acid [Sigma, St.Louis, MO, U.S.A]) for 14 days (differentiation stage). Next, cells were incubated in mineralization medium (differentiation medium supplemented with 2.5 mg/ml of ITS [insulin-transferrin-selenium, Sigma], 5 mM beta-glycerophosphate [Sigma] and 10^{-8} M dexamethasone [Sigma]) for another 14 days (mineralization stage).

Real time RT-PCR

At the end of each differentiation stage, total RNA was isolated from HCDCs and PDL cells using Perfect RNA™ (Eppendorf Scientific, Inc., Westbury, NY, U.S.A) and converted to cDNA using SuperScript™ reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A) as specified by manufacturers. Real time RT-PCR was performed with LightCycler - FastStart DNA Master SYBR Green I kit (Roche Applied Science, Indianapolis, IN, U.S.A). Relative abundances of specific mRNA sequences were determined by comparison with a standard curve generated by serial dilution of a cDNA sample containing abundant target sequences and normalized to the expression of GAPDH. All

PCR reactions were performed in triplicate. Human BMP specific primers were designed based on published mRNA sequences (GenBank). Sequences of primers used are as follows: BMP-2 (sense 5' TTGCGGCTGC TCAGCATGTT 3', antisense 5' TTCCGAGAACAGATGCAAGATG 3'), BMP-3 (sense 5' AGGTCTCTGAACACATGCTG 3', antisense 5' ATCAAGCTTACAGGGACACC 3'), BMP-4 (sense 5' AGCC ATGCTAGTTTGATACC 3', antisense 5' TCAGGGATGCTGCT GAGGTT 3'), BMP-5 (sense 5' AGACAATCATGTTCACTCC AGTT 3', antisense 5' AGCTGTAAGCCCAAATTATTCTGG 3'), BMP-6 (sense 5' ACATGGTCATGAG CTTTGTGA 3', antisense 5' GTAGAGCGATTACGACTCTGT 3'), BMP-7 (sense 5' CAGCCTGCAAGATAGCCATT 3', antisense 5' AATCGGATCTCTTCCTGCTC 3'), BMP-8 (sense 5' CGTGC AGCGCGAGATCCTGG 3', antisense 5' GCCTCTATGTGGA GACTGAG 3') and GAPDH (sense 5' ACCACAGTCCATG CCATCAC, antisense 5' TCCACCACCCTGTTGCTGTA 3').

Results

The gene expression of BMP-2 through BMP-8 by human cementum-derived cells was evaluated by employing real time RT-PCR technique.

Fig. 1-5 shows most of the BMPs examined were expressed except BMP-7 and BMP-8. The relative expression of BMP-3 mRNA (Fig. 2) was the highest while that of BMP-6 mRNA (Fig. 5) was the lowest. In the HCDCs, all of these genes was expressed higher level than in the PDL cells.

In terms of pattern of expression, in BMP-2, 5 and 6 (Fig. 1,4,5), the relative expression of each BMP was decreased as differentiation advanced. In BMP-4 (Fig. 3), the relative expression was increased from proliferation stage to differentiation stage and then decreased from differentiation stage to mineralization stage. In BMP-3 (Fig. 2), the relative expression showed opposite pattern of BMP-2, 5 or 6.

Discussion

Although the BMPs play important roles in skeletal development and in the differentiation of osteoblasts and chondroblasts, little is known about their roles in the differentiation of cementoblasts and cementum homeostasis. Using human cemetum-derived cells (HCDCs), we found that BMPs (BMP 2-6) showed dynamic expression patterns in these cell strains and relative expressions of BMPs in HCDCs were higher than in PDL cells. This result indicates BMP 2-6 may play certain roles in cementum physiology.

BMPs were originally isolated from demineralized bone extract (Urist, 1965) and induced the ectopic formation of new cartilage and bone when implanted in adult animals (Wozney *et al.*, 1988, Wang *et al.*, 1990). Among them, BMP-3 is the most abundant BMP in demineralized bone, accounting for approximately 65% of the total (Luyten *et al.*, 1989, Wozney

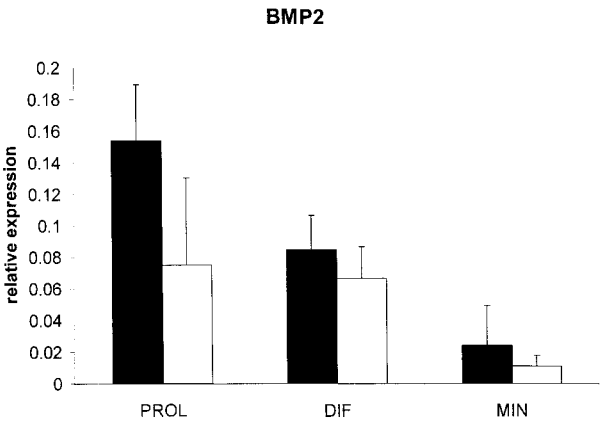

Fig. 1. BMP-2 expression (mean + standard error of the mean) by HCDCs and PDL cells was determined using real time RT-PCR and normalized to GAPDH expression. All PCR reactions were performed in triplicate. (■: HCDCs, □: PDL cells, PROL: proliferation stage, DIF: differentiation stage, MIN: mineralization stage)

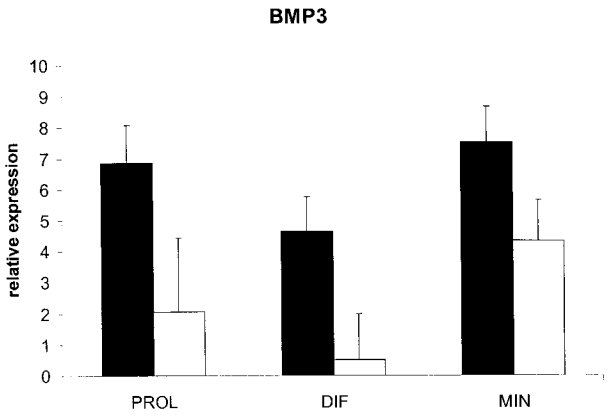

Fig. 2. BMP-3 expression (mean + standard error of the mean) by HCDCs and PDL cells was determined using real time RT-PCR and normalized to GAPDH expression. All PCR reactions were performed in triplicate. (■: HCDCs, □: PDL cells, PROL: proliferation stage, DIF: differentiation stage, MIN: mineralization stage)

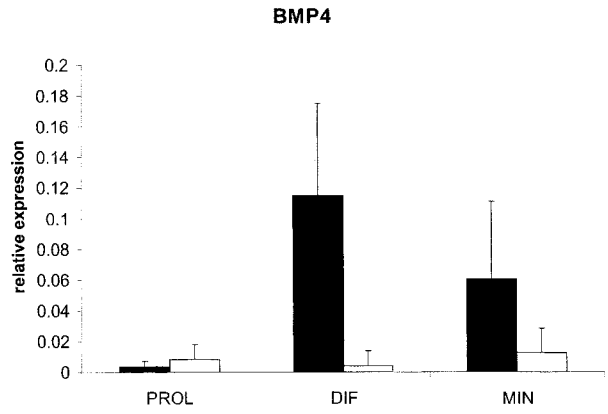

Fig. 3. BMP-4 expression (mean + standard error of the mean) by HCDCs and PDL cells was determined using real time RT-PCR and normalized to GAPDH expression. All PCR reactions were performed in triplicate. (■: HCDCs, □: PDL cells, PROL: proliferation stage, DIF: differentiation stage, MIN: mineralization stage)

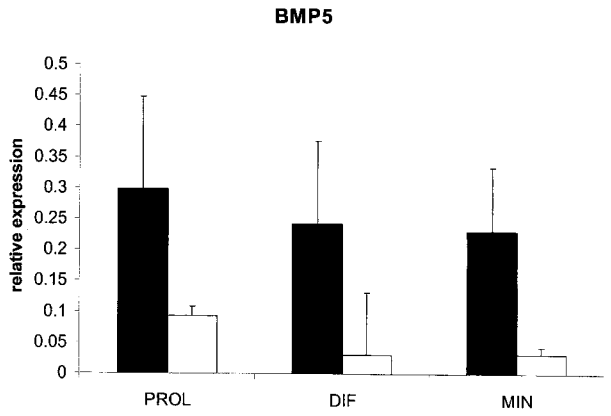

Fig. 4. BMP-5 expression (mean + standard error of the mean) by HCDCs and PDL cells was determined using real time RT-PCR and normalized to GAPDH expression. All PCR reactions were performed in triplicate. (■: HCDCs, □: PDL cells, PROL: proliferation stage, DIF: differentiation stage, MIN: mineralization stage)

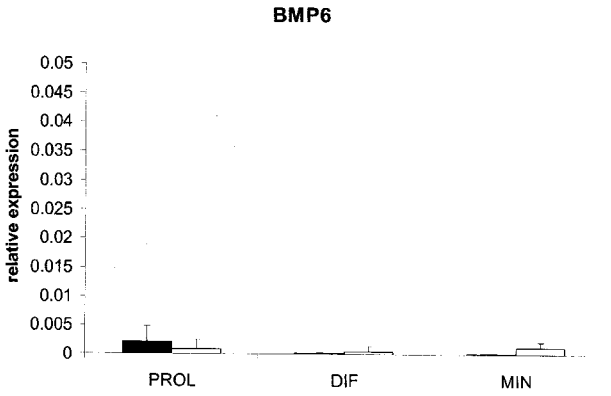

Fig. 5. BMP-6 expression (mean + standard error of the mean) by HCDCs and PDL cells was determined using real time RT-PCR and normalized to GAPDH expression. All PCR reactions were performed in triplicate. (■: HCDCs, □: PDL cells, PROL: proliferation stage, DIF: differentiation stage, MIN: mineralization stage)

and Rosen, 1993). In this study, the relative expression of BMP-3 by HCDCs was much higher than other BMPs when assessed by real time RT-PCR and similar patterns of BMP expressions were observed in cultured osteoblastic cell models (including primary human osteoblasts, BMSC [data are not shown]). Hence, it is conceivable that BMP-3 may be an important regulator in bone and cementum homeostasis. However, current information on the role of BMP-3 in bone is contradictory. Although osteogenin (BMP3 purified from bone) is osteoinductive, rhBMP3 has no osteogenic activity (Takao *et al.*, 1996). Daluiski *et al.*(2001) generated BMP-2 or BMP-3 producing cells by infection these genes into osteoprogenitor cells employing retroviral system to investigate their effects in osteoprogenitor cells. They reported implantation of BMP-2-producing cells into quadriceps muscles of mice causes heterotopic bone formation, whereas implantation of BMP-3-producing cells did not. Moreover, BMP-3 blocked BMP-2-mediated differentiation of osteoprogenitor cells into osteoblasts. Therefore, it is difficult to predict whether BMP-3 produced by cementoblasts takes positive effects or negative effects on cementum formation and regeneration. Further research to confirm the effects of BMP-3 is pending. According to the results of the further research, we may be able to use rhBMP-3 as a positive or negative regulator of cementum formation and regeneration.

The expression pattern of each BMP in the course of differentiation was different. On average, there was a tendency for most BMPs to be expressed at higher levels earlier in the course of differentiation than at later stages, that is, the relative expression was decreased before mineralization occurred in culture except that of BMP-3 which showed opposite pattern. Hence, we can hypothesize here that BMP-2, 4, 5 and 6 may be involved in the early stage of cementoblast differentiation or cementum regeneration and BMP-3 may play a different role from others. There are approximately 15-20 BMP genes in human. BMPs fall into several subgroups based on their sequence homology (Takao *et al.*, 1996). BMP-2 and BMP-4 are very closely related (86% amino acid identity in mature region). BMP-5, 6, 7 and BMP-8 are also closely related to each other (61-79%). However, BMP-3 is quite different from other BMPs. This feature is in accordance with our results. Therefore, further investigation to corroborate our hypothesis is needed.

In summary, most of the BMPs examined were expressed by HCDCs in this study, indicating BMPs secreted by cementoblasts may be involved in cementum homeostasis. However, similar patterns of BMP expressions were observed in cultured osteoblastic cell models (including primary human osteoblasts, BMSC), indicating that cementoblasts are closely related to osteoblasts. Therefore, BMPs are likely to be involved in regulation of more general features of cemento-blastic cells (probably related to its mineralizing tissue forming phenotype) rather than those unique to cementum.

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