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Acceleration Effect of Human Recombinant Bone Morphogenetic Protein-2 on Differentiation of Human Pulp Cells Into Odontoblasts

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Predictable pulp capping procedures remain problematic, possibly because of the lack of appropriate stimulating factors for dentin formation. The present study examines the ability of one such stimulating factor, bone morphogenetic protein-2, to accelerate the differentiation of human dental pulp cells into odontoblasts. The number and morphology of cells between groups treated with 0 and 100 ng/ml of human recombinant bone morphogenetic protein-2 (rhBMP-2) did not significantly differ. However, ALPase activity (a marker for biomineralization) in the group stimulated with rhBMP-2 was more than double that of the control group. We then measured the expression of mRNA encoding dentin sialophosphoprotein (DSPP) as a marker of odontoblasts in rhBMP-2–stimulated human pulp cells using a quantitative polymerase chain reaction. The expression of DSPP mRNA in cells stimulated for 24 h by 1000 ng/ml of rhBMP-2 was approximately 20-fold and 5-fold higher than that by stimulated by 10 and 100 ng/ml, respectively. These findings show that rhBMP-2 promoted the differentiation of human dental pulp cells into odontoblasts but did not affect cell proliferation, suggesting that rhBMP-2 may have therapeutic utility in vital pulp therapy.

Because the vitality and function of the dentin-pulp complex directly affects tooth conservation, various vital pulp therapies have been developed. Calcium hydroxide-based agents are most frequently used as direct pulp capping agents because they can stimulate pulpal tissue to produce reparative dentin. However, these agents result in permanent necrotic tissues produced by the high alkalinity (pH 11 to 12), and the newly formed secondary dentin is often porous. Thus, secondary infection of the pulp tissue can develop. Furthermore, low mechanical strength, poor adhesion between dentin and dentin adhesion systems, and the potential pulpal resorption are potential disadvantages of these agents (1, 2). Therefore, a biocompatible, bioactive agent that rapidly induces dentin formation is required to improve the clinical success of vital pulp therapy.

Bone morphogenetic protein (BMP) is a growth factor belonging to the TGF-β superfamily. It is a good candidate for vital pulp therapy (3, 4) because it is responsible for bone induction and has chondrogenetic activity (5). Demineralized dentin matrix also contains BMP. Under pathologic conditions such as carious lesions, the secretory activity of odontoblasts is stimulated to produce dentin via a process that may be exerted by signaling molecules such as BMP liberated from the dentin during demineralization (6). In contrast, deep cavity preparation leads to odontoblast disintegration, and then odontoblast-like cells, possibly derived from dental pulp stem cells capable of multilineage differentiation, terminally replace mature odontoblasts and induce reparative dentin formation (7). Activation of stem cells in human pulp represents a potential cellular approach to pulp capping (8–10). Our hypothesis is that the differentiation of dental pulp stem cells into odontoblasts can be accelerated by signaling molecules as a pulp capping strategy, particularly when the signaling molecules contain BMP.

Hydroxyapatite forms the main inorganic portion of dentin, whereas type I collagen constitutes most of the organic portion. Small amounts of the noncollagenous proteins that are found in the extracellular matrix of bone such as osteonectin, osteopontin, osteocalcin, bone sialoprotein, and bone matrix protein-1 are also found in dentin (11). Dentin sialoprotein and dentin phosphoprotein (DPP) are specific to dentin and are the result of a common transcript for dentin sialophosphoprotein (DSPP) (12). Therefore, DSPP is considered a good marker for odontoblasts.

This study measures DSPP gene expression using quantitative polymerase chain reaction (PCR) to determine the potential of BMP-2 to accelerate the differentiation of human dental pulp cells into odontoblasts.

MATERIALS AND METHODS

In brief, we derived the human recombinant BMP-2 (rhBMP-2) variant from transfected *Escherichia coli*. The N-terminal 12 amino acid residues (MAKHKQRKRLKS) of human BMP-2 constitute a heparin-binding site that does not induce bone formation. These residues are substituted with the N-terminal 13 amino acid residues (MAPTSSSTKKYQL) of human interleukin-2 in the rhBMP-2 variant derived from *E. coli* (13).

We obtained written informed consent from three patients 19 to 21 years old to extract the third molars during normal treatment. The extracted teeth were immediately placed into calcium-free and magnesium-free phosphate buffered saline (PBS; Invitrogen Corp., Carlsbad, CA) and cracked open. Extruded pulp tissues were cut into 2-mm^3 cubes using a dental surgical knife and incubated at 37°C in 100-mm culture dishes (Iwaki, Chiba, Japan) containing α-modified minimum essential medium (Invitrogen Corp.) supplemented with 15% fetal bovine serum (Multiser Scientific Ltd., Melbourne, Australia), antibiotics (100 U/ml of penicillin G potassium and 100 μg/ml of streptomycin sulfate), and 82 μg/ml L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako Chemical Co., Osaka, Japan) in a humidified atmosphere of 95% air and 5% carbon dioxide. At subconfluence, the cells were subcultured under the same conditions with medium changes at 2-day intervals. We examined cells from passages 4 through 7.

Recombinant human BMP-2 (100 ng/ml) was added to the cells in 24-well plates (Iwaki). The shape of the cells changed at 1, 5, 10, 15, and 20 days thereafter, and then the cells were counted and ALPase activity was measured. The activity of ALPase is considered essential for biomineralization. The cells were washed three times with phosphate buffered saline (−) and sonicated with 1 ml of 0.1-M Tris buffer (pH 7.2) containing 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO) for 30 s on ice. Cellular ALPase activity was assayed by the method of Lowry *et al*. (14) using p-nitrophenyl phosphate as a substrate. The enzyme activity is expressed as micromoles of p-nitrophenyl produced per minute per milligram of protein. The protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad, New York, NY) with albumin as the standard. After reaching confluence in 60-mm culture dishes, rhBMP-2 (0, 10, 100, or 1000 ng/ml) was added to the cells with serum-free medium. After 30 min and 1, 6, and 24 h, the cells were collected with TRIzol Reagent (Invitrogen). Total cellular RNA was reverse-transcribed using SUPERSCRIPT II (Invitrogen). We examined the expression of DSPP mRNA as a marker of odontoblasts in human pulp cells stimulated by rhBMP-2 using quantitative and conventional PCR. Quantitative PCR assay was performed using a LightCycler and the double-stranded DNA dye SYBR Green I (Roche Diagnostics, Mannheim, Germany) (15). The final concentration of $MgCl_2$ was 3 mM. A standard curve was constructed by refining PCR products using a High Pure PCR Product Purification Kit (Roche Diagnostics) followed by 10-fold dilutions. Melting curve analysis showed that the melting temperature of a single peak was 82°C. In addition, the final PCR product on 2% agarose gels migrated as a single band with a sequence identical to that of DSPP. The copy number of DSPP mRNA was divided into that of GAPDH for quantitation. Conventional PCR simultaneously proceeded in a Takara PCR Thermal Cycler Personal (Takara, Kyoto, Japan) using Fast-Start DNA Taq Polymerase (Roche Diagnostics). The oligonucleotide sequences used for both PCR procedures were as follows: DSPP, 5'-CAG CCA AAG ATA GAG GAC-3' (upper primer) and 5'-GGG ACC CTT GAT TTC TAT-3' (lower primer); GAPDH, 5'-TGA ACG GGA AGC CTC ACT GGG-3' (upper primer) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (lower primer).

The results for cell numbers and ALPase activities were analyzed by Student's *t* test and are expressed as means ± SD. Expression of the dentin sialophosphoprotein gene was analyzed by one-way analysis of variance with Tukey's multiple comparison test. Differences at $p < 0.01$ were considered statistically significant.

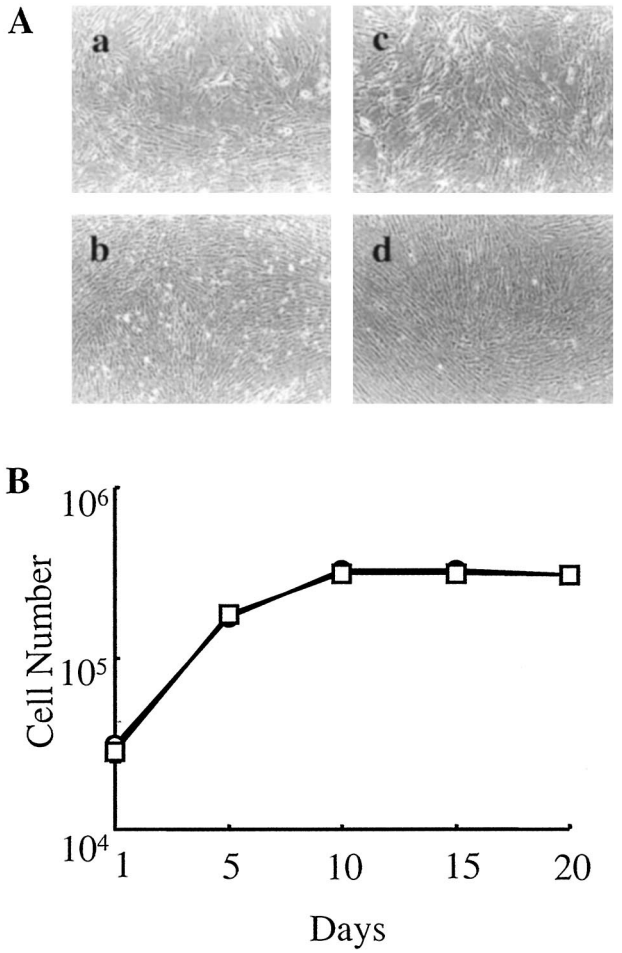

FIG 1. Effects of 100 ng/ml of rhBMP-2 on morphology and proliferation of human dental pulp cells. (*A*) Phase-contrast features of human dental pulp cells at 5 (*a*) and 20 (*b*) days of culture with, and at 5 (*c*) and 20 (*d*) days of culture without rhBMP-2 (original magnification × 100). (*B*) Time-course changes in numbers of cells cultivated with (*squares*) and without (*circles*) rhBMP-2. Data represent means ± SD in six samples in three separate experiments. No effect significantly differed between experimental and control values.

RESULTS

Morphologic Analysis and ALPase Activity

Human dental pulp cells with and without exposure to rhBMP-2 reached confluence on day 7 and remained fibroblast-shaped throughout the experimental period (Fig. 1). The numbers of cells

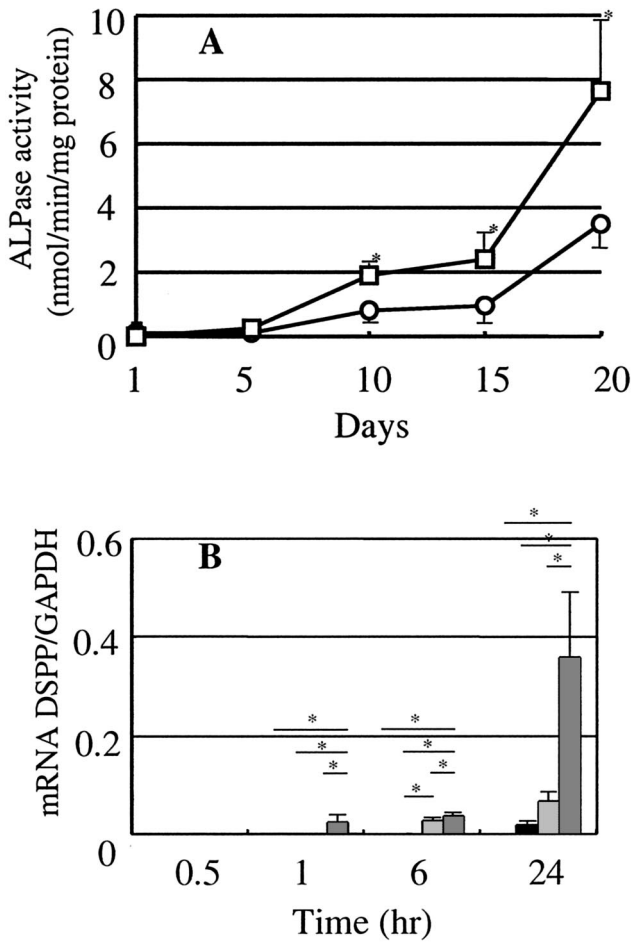

FIG 2. Effects of rhBMP-2 on differentiation of human dental pulp cells. (*A*) Effect of rhBMP-2 on ALPase activity in human dental pulp cells. Cells were cultured with 100 ng/ml of rhBMP-2 (*squares*) or without rhBMP-2 (*circles*). Data represent means ± SD in six samples in three separate experiments. Significantly different from control values (*$p < 0.01$). (*B*) Graph of DSPP mRNA copy number determined using LightCycler. Blue, green, and red bars indicate values from 10, 100, and 1000 ng/ml of rhBMP-2, respectively. Expression of DSPP mRNA at 0 ng/ml of rhBMP-2 (*white bar*) could not be quantified. Data represent means ± SD in six samples in three separate experiments. Significant difference at the level of *$p < 0.01$.

between the groups with and without rhBMP-2 did not significantly differ (Fig. 1). On the other hand, ALPase activity more than doubled in the presence of rhBMP-2 (Fig. 2).

Analysis of DSPP mRNA Expression Using Quantitative PCR

Human dental pulp cells without rhBMP-2 did not express DSPP mRNA throughout the experimental period. The expression of DSPP mRNA in the cells incubated for 30 min with rhBMP-2 could not be quantified because the values were below the limits of detection (Fig. 2). Cells expressed DSPP mRNA after 1 h only when stimulated with 1000 ng/ml of rhBMP-2 and after 6 h when stimulated with either 100 or 1000 ng/ml of rhBMP-2 (Fig. 2). The results obtained using the LightCycler showed that after 24 h of stimulation, 20-fold more DSPP mRNA was expressed in the cells

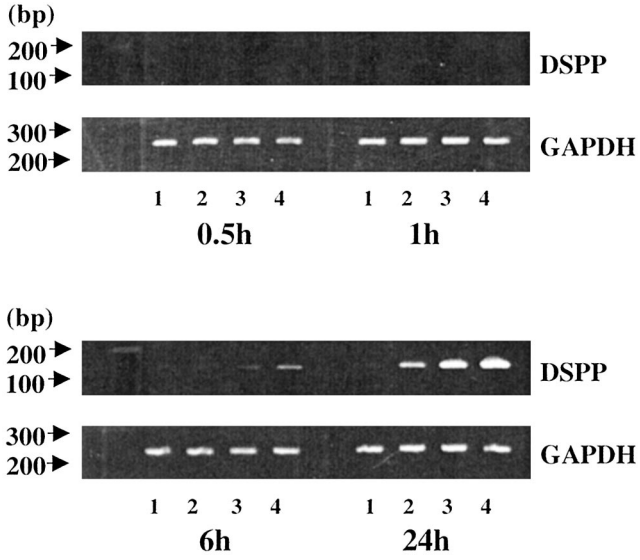

FIG 3. Expression of DSPP mRNA in human dental pulp cells cultured without (*lane 1*) and with 10 (*lane 2*), 100 (*lane 3*), and 1000 ng/ml (*lane 4*) of rhBMP-2 determined using conventional PCR. Products of DSPP and GAPDH were 133 and 285 bp, respectively. Data are representative of six samples in three separate experiments.

stimulated with 1000 ng/ml of rhBMP-2 compared with that by stimulated with 10 and 100 ng/ml of rhBMP-2 (Fig. 2). Moreover, DSPP mRNA expression in the cells was increased by rhBMP-2 in a concentration-dependent and time-dependent manner in conventional PCR (Fig. 3).

DISCUSSION

Dental pulp cells can differentiate into odontoblasts that synthesize and assemble reparative dentin to counter damage by noxious stimuli such as caries and operative procedures (16). We and others have hypothesized that pulpal growth and differentiation factors and their cell surface receptors may be involved in pulpal wound healing. BMPs may induce tissue formation by triggering responsive cells to initiate signaling cascades.

Bone morphogenetic proteins are known to activate multiple signaling pathways leading to enhanced biomineralization. BMPs bind to receptors with intrinsic serine/threonine kinase activity and activate Smads, which are signal transducer molecules. Bound BMPs then oligomerize with the common mediator Smad4 and translocate to the nucleus, where they direct transcription to initiate the cellular response to BMP (17). Cbfa1/Runx2 is an essential transcription factor for osteoblast and odontoblast differentiation. Importantly, the binding sites for Cbfa1/Runx2 have been identified in the regulatory elements of the DSPP gene, which encodes a matrix protein expressed during odontogenesis. Thus, Cbfa1/Runx2 may play a role in the regulation of DSPP gene expression (17). The gene expression of DSPP may have been upregulated by rhBMP-2 and mediated through Cbfa1/Runx2 in the present study.

A population of putative postnatal stem cells in human dental pulp, has recently been identified (8–10). The most striking feature of dental pulp stem cells is their ability to regenerate a dentin-pulp–like complex that is composed of mineralized matrix with tubules lined with odontoblasts and fibrous tissue containing blood

vessels. The differentiation of dental pulp stem cells into odontoblasts in vitro was stimulated in the present study by rhBMP-2. Therefore, a cellular approach to pulp capping using dental pulp stem cells and rhBMP-2 is feasible.

We found that 100 ng/ml of rhBMP-2 affected neither the proliferation of human dental pulp cells nor the cell morphology. On the other hand, rhBMP-2 stimulated ALPase activity in pulp cells. Thus, rhBMP-2 functioned as a differentiation factor to human dental pulp cells rather than a growth factor in the present study. A similar effect of 50 ng/ml of BMP-2 on proliferation and differentiation has been reported in bovine pulp cells (18). The activity of ALPase in odontoblasts and subodontoblastic cells is higher than in undifferentiated mesenchymal cells. Because ALPase activity in the subodontoblastic layer is highest in the pulp tissue, the activity of this enzyme must be prerequisite for pulp cells to differentiate into odontoblasts (19). However, ALPase activity can indicate cells other than preodontoblasts or odontoblasts.

We therefore used quantitative PCR to investigate DSPP gene expression by human dental pulp cells as a differentiation marker of odontoblasts in the presence of rhBMP-2. The expression of DSPP mRNA was increased in a dose-dependent manner when pulp cells were incubated with 10, 100, and 1000 ng/ml of rhBMP-2 for 24 h. Moreover, rhBMP-2 promoted DSPP mRNA expression in a time-dependent manner. A small amount of DSPP mRNA has been detected in young odontoblasts secreting predentin in rodent incisors (20). The expression of DSPP mRNA concomitantly increased in mature odontoblasts with the start of dentin mineralization, which is considered a terminal phenotypic marker of mature odontoblasts (20). The immunolocalization of dentin sialoprotein and DPP in young and mature odontoblasts and in dentin suggests that these proteins are important in dentinogenesis and possibly in dentin mineralization (21). We also demonstrated that DPP induces hydroxyapatite formation when covalently cross-linked to type I collagen fibrils in vitro, and that the potential of DPP for hydroxyapatite nucleation is similar to that of hydroxyapatite on crystal growth in vitro (22–24). However, von Kossa staining in the present study did not identify ECM mineralization in pulp cells in the presence of rhBMP-2 (data not shown), although DSPP gene expression was intense. This may be a result of the short-term nature of the experiment.

The present study is the first to use quantitative PCR to show that rhBMP-2 accelerates DSPP gene expression in vitro. The results suggest that rhBMP-2 promotes the differentiation of human dental pulp cells into odontoblasts. Thus, rhBMP-2 is a promising biocompatible pulp capping material that can induce rapid reparative dentin formation. Further development of a biocompatible drug delivery system using rhBMP-2 is necessary.

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