The Distinct Roles of Growth/Differentiation Factor-5 in Cell Proliferation and Odontoblast Differentiation from Dental Pulp Cells

Y Hatakeyama, Y Maruya, J Hatakeyama, K Oka, E Tsuruga, T Inai, Y Sawa

Citation

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Abstract

Growth/differentiation factor-5 (GDF-5) belongs to the bone morphogenetic protein (BMP) family, which is expressed in dental pulp tissues. To date, various studies have reported that BMP-family growth factors can induce cells isolated from dental pulp tissues to differentiate into odontoblasts. Nevertheless, the effect of GDF-5 on odontoblast differentiation from dental pulp tissues remains unknown. In this study, we assayed the effect of exogenous mouse recombinant GDF-5 on cell proliferation of dental pulp cells and determined the expression levels of odontoblast differentiation marker genes in the cells of isolated mouse dental pulp tissues in the presence of GDF-5. There were no significant differences in dental pulp cell proliferation patterns related to the absence or presence of GDF-5 after seven days of culture. Odontoblast marker gene levels, on the other hand, were significantly elevated after seven days of culture in the presence of GDF-5. GDF-5 also increased the gene expression and enzyme activity of alkaline phosphatase. Furthermore, dental pulp cells cultured in the presence of GDF-5 formed alizarin red-positive nodules after 10 days of culture. These results indicate that, although GDF-5 may have no effect on cell proliferation in isolated dental pulp tissue, it could promote odontoblast differentiation.

INTRODUCTION

Tooth development consists of multiple steps of reciprocal signaling interaction between the epithelium and the mesenchymal cells. Dental epithelium and mesenchymal cells each differentiate into a particular major cell type which produces a distinctive hard tissue; specifically, enamel is deposited by ameloblasts derived from the epithelium, while dentin is deposited by odontoblasts derived from mesenchymal cells. The odontoblasts are differentiated from the dental papilla, which remains a soft connective tissue in the tooth interior also known as the dental pulp, even after development is complete. The cells in the dental pulp comprise a heterogeneous mixed population, made up of odontoblasts, fibroblasts, stem cells, and macrophages and other immunocompetent cells. To date, various studies have reported that cells isolated from the dental pulp of various animals can be induced to differentiate into cells of the odontoblastic phenotype by means of various chemicals, growth factors and signaling molecules both in vitro and in vivo (reviewed in Nakashima and Akamine, 2005). Yet it remains poorly understood how these factors can induce the various types of cells in the dental pulp to engage in a harmonized and coordinated process of dentin regeneration.

Growth/differentiation factor-5 (GDF-5) is a member of the bone morphogenetic protein (BMP) family, which is a subgroup of the transforming growth factor-β (TGF-β) superfamily. Along with other BMPs, including BMP-2, -4 and -7, which are well known as potent osteoinductive growth factors, GDF-5 plays important roles in the development of bones, cartilage and tendons, as evidenced by the tendency for the gene and protein expression levels of GDF-5 to increase over time in these tissues. In addition, development and genetic studies have shown that GDF-5 null mutation or transgenic mice exhibit abnormal growth patterns or overgrowth of limbs, long bones, cartilage, joints and digits (Storm et al., 1994, Francis-West et al., 1999, Tsumaki et al., 2002). It has also been reported that the GDF-5 gene is expressed in dental sac, periodontal ligament, dental pulp cells, and odontoblasts during tooth development (Morotome et al., 1998, Nakashima et al., 1998, Sena et al., 2003). A previous in-vitro study on the role of GDF-5 in periodontal ligament cells has shown that exogenous GDF-5 promotes cell proliferation while suppressing the activity of

alkaline phosphatase (ALP), which is closely related to tissue calcification and which is secreted during the formation of tissue calcifications. In dental pulp cells, however, the functions of GDF-5 are still unknown. Here, we investigated the effect of GDF-5 on the proliferation of dental pulp cells and their differentiation into odontoblasts by assaying the expression levels of odontoblast marker genes. These findings are expected to improve our knowledge of the role of GDF-5 in dental tissue development and to provide information that may be useful in the application of GDF-5 as a molecular manipulator in dental tissue regeneration.

MATERIALS AND METHODS

CELL CULTURE

Mouse dental pulp cells were isolated from the first molars of one-week-old mice. Specifically, first molars of one-week-old mice were dissected and dental pulp was isolated with instruments under microscopy. After dissection, the apical portions of dental pulp samples were removed to prevent periodontal fibroblast contamination, and the remaining crown portions were minced into small pieces. These small dental crown pulp pieces were incubated in phosphate saline buffer (PBS) containing 1.0% collagenase and 0.4% Trypsin at 37˚C for 15 minutes. Isolated cells were washed in Hanks balanced salt solution and were re-suspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Equitech-Bio Inc., Tokyo, Japan) and antibiotics (100 U/ml of penicillin-G and 100 mg/ml of streptomycin). Cells were seeded on a normal plastic cell culture plate at 37˚C in a humidified atmosphere of 5% CO_2 in air and were used for each experiment after two or four passages.

CELL PROLIFERATION ASSAY

The proportional numbers of cells were counted using a Cell-Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), which depends on measuring a highly water-soluble formazan dye produced from tetrazolium salts. Dental pulp cells were seeded at 1.0 x 10^3 cells per well of a 96-well plate and media containing various concentrations of recombinant mouse GDF-5 (R&D Systems, Minneapolis, MN, USA) and 50 ng/ml of recombinant mouse basic fibroblast growth factor (bFGF, R&D Systems) were added. At each indicated time point, namely, after one, two, three, four and seven days of cell culture, cells were incubated with a counting reagent for one hour, according to the kit manufacturer's instructions, and the relative cell number was determined by measuring light absorbance of a formazan dye product in the cultures at a wavelength of 450 nm.

ASSAY FOR ALKALINE PHOSPHATASE (ALP) ACTIVITY

Dental pulp cells were seeded on 24-well cell culture plates and cultured in the absence or presence of 500 ng/ml GDF-5 for seven days after cell confluence was achieved; the medium contained 10% FBS, 50 g/ml ascorbic acid, 100 nM Dexamethasone and 10 mM beta-glycerophosphate (Sigma Aldrich, St. Louis, MO, USA). Quantitative analysis of ALP activity was performed as previously described with minor modifications (Aoki, 2001). Briefly, cells were washed with Tris buffer saline (TBS) (50 mM Tris, pH 7.4, and 0.15 M NaCl), extracted with a TBS-based lysis buffer containing 0.5 mM $MgCl_2$ and 0.1% Triton X-100, and stored at -20˚C until assayed. Alkaline phosphatase activity was measured using p-nitrophenyl phosphate at a final concentration of 10 mM (Sigma-Aldrich) as a substrate in 0.7 M 2-amino 2 methyl 1-propanol and 6.7 mM $MgCl_2$, pH 10.3. After 10 min of incubation at 37˚C, the reaction was stopped with 0.5 N NaOH and absorbance was measured at 405 nm. Alkaline phosphatase activity was measured as the amount of p-nitrophenol produced in one nmol/min/mg protein. Protein was measured using the Pierce BCA Protein Assay Reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA) with bovine serum albumin as a standard according to the manufacturer's instructions. Three independent experiments were performed in each group.

ALIZARIN RED STAINING

To identify matrix calcification by dental pulp cells, we used Alizarin Red S staining as described previously with minor modifications (Hessle et al., 2002). Briefly, dental pulp cells were cultured under conditions similar to those used in the assay for ALP activity, described above. After 10 days, cells were briefly rinsed with Tris-buffered saline (TBS), then fixed in ice-cold methanol for 20 min. After fixation, cells were washed three times with TBS and rinsed with deionized water, then stained with 0.5% (vol/vol) Alizarin Red S, pH 5.0, for 10 min at 23˚C. Cultured cells were then rinsed five times with deionized water and observed under bright field microscopy.

GENE EXPRESSION ASSAY

Dental pulp cells were cultured under conditions similar to those used in the assay for ALP activity after cell confluence had been achieved. At each indicated time point during the

seven-day culture period, total RNA was isolated from dental pulp cells using an RNeasy Mini Kit (Qiagen, Inc., Tokyo, Japan) according to the manufacturer's instructions. The expression levels of genes related to osteogenic or odontogenic differentiation, ALP, bone sialoprotein (BSP), collagen type 1 alpha 2 (Col1α2), osteopontin (OPN), osteonectin (OSN), osteocalcin (OSC), dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1) were assayed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Primer sequences of each gene were designed as previously described (Jadlowiec et al., 2004, Wu et al., 2010, Sumita et al., 2010) except for Col1α2, whose primer was designed as follows: Gene Accession number NM_007743, Forward, 5' AGC GGT GAA GAA GGA AAG 3', Reverse, 5' CTT TCC TTC TTC ACC GCT 3', fragment size 510 bp. Gene expression levels were evaluated relative to those of mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as described previously (Hatakeyama et al., 2004).

STATISTICAL ANALYSIS

Numerical data were analyzed by a computer software package for statistical analysis (Excel, Microsoft Co., Tokyo, Japan). Statistical significance was determined using Fisher's test.

RESULTS

In order to analyze the function of GDF-5 in dental pulp differentiation systematically, we analyzed the effect of GDF-5 on cell proliferation. Dental pulp cells were cultured in the absence or presence of GDF-5 at 500 ng/ml for each cell culture period (Fig. 1). This dose of GDF-5 was tested because similar doses have been used previously to elicit mesenchymal cell proliferation (Hatakeyama, et al., 2004). Cell number was measured in the form of light absorbance at a wavelength of 450nm of a formazan dye product in the culture, and relative cell number in each group was recorded as a percentage of the initial value. Cell numbers increased throughout the seven-day culture period both in the absence (Fig. 1, Control) and in the presence of GDF-5 (Fig. 1, GDF-5), and there was no significant difference in cell number at any of the measurement points (one-day culture: Control, 117.3 ± 2.4%, GDF-5, 117.9 ± 4.1%, two-day culture: Control, 120.2 ± 4.3%, GDF-5, 125.9 ± 3.0%, four-day culture: Control, 134.0 ± 6.3%, GDF-5, 129.5 ± 4.3%, seven-day culture: Control, 146.0 ± 8.0%, GDF-5, 152.3 ± 4.5%). Since it has been reported that exogenous recombinant bFGF increases the number of dental pulp cells derived from human third molars in vitro (Shiba et al., 1998), we assayed the effect of bFGF on the proliferation of cells derived from immature mouse dental pulp. We found that exogenous recombinant mouse bFGF significantly increased the number of dental pulp cells at all culture periods including one-day culture (Control: 117.3 ± 2.4%, bFGF: 136.4 ± 9.0%; Fig. 1). These results indicate that dental pulp cells derived from the molars of one-week-old mice exhibit increased cell proliferation in the presence of exogenous bFGF but not in the presence of GDF-5.

Next, we assayed the effect of GDF-5 on the expression of several odontoblast marker genes, namely, BSP, OPN, OSN, OSC, DSPP and DMP-1, by RT-PCR. The expression level of each gene was observed at the beginning of dental pulp cell culture (Fig. 2, Initial). From this initial time point, the expression of each gene increased significantly over the seven-day culture period in the absence of GDF-5 (Fig. 2, Control), but, except for DMP-1 (data not shown), the expression of each gene increased significantly further in the presence of GDF-5 (Fig. 2, GDF-5) than in its absence (Fig. 2, Control).

Since ALP is a marker gene for odontoblast cell differentiation, a marker enzyme for dentinogenesis, and particularly related to the calcification of extracellular matrices (Lin et al., 2011, Ikeda et al., 2011), we examined whether GDF-5 promoted ALP gene expression and enzyme activity in dental pulp cells. Dental pulp cells were cultured for seven days in the absence or presence of 500 ng/ml GDF-5 after cell confluence was achieved. The gene expression level of ALP was assayed by RT-PCR and gene expression was normalized against GAPDH. ALP gene expression was observed at the beginning of dental pulp cell culture when cell confluence had been achieved (Fig. 3A, Initial). Compared to this initial value, ALP gene expression was elevated significantly after seven days of culture in an odontoblast differentiation medium containing ascorbic acid, dexamethasone and beta-glycerophosphate but not containing GDF-5 (Fig. 3A, Control); in the presence of GDF-5, however, ALP gene expression was significantly higher than in the absence of GDF-5 (Fig. 3A, GDF-5).

To measure ALP enzyme activity quantitatively, we measured light absorbance using p-nitrophenyl phosphatase as a substrate, and normalized the resulting ALP activity values to total protein levels in each sample. In the absence of GDF-5, ALP activity was significantly greater (Fig. 3B, Control) than it had been at the beginning of the culture

period (Fig. 3B, Initial). In the presence of GDF-5, however, ALP enzyme activity was significantly higher (Fig. 3B, GDF-5) than it was in the absence of GDF-5 (Fig. 3B, Control). Alizarin red staining showed that 500 ng/ml of GDF-5 induced alizarin red-positive nodules after 10 days of culture (Fig. 3D). In the absence of GDF-5, on the other hand, there were no alizarin red-positive nodules (Fig. 3C).

Figure 1

Fig. 1 GDF-5 did not affect dental pulp cell proliferation. Dental pulp cells were cultured in the absence (Control) or presence of GDF-5 at 500 ng/ml (GDF-5) for each culture period after one-day culture for initial cell attachment (Int). Cell number was measured by light absorbance at a wavelength of 450 nm of a formazan dye product in the culture, and the relative O.D. value of each group was designed as a percentage of the initial value. There was no significant difference in cell number between GDF-5-treated and control cells. bFGF at 50 ng/ml increased dental cell numbers at each cell culture period. =8 in each group; * < 0.05 compared with control cells cultured for the same length of time.

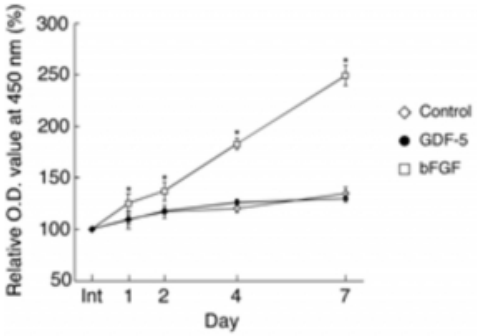

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Figure 2

Fig. 2 GDF-5 promoted the expression of odontoblast marker genes. Dental pulp cells were cultured in the absence (Control) or presence of GDF-5 at 500 ng/ml (GDF-5) for seven days after cell confluence was achieved (Initial). The expression levels of odontoblast marker genes, including bone sialoprotein (BSP, A), Collagen type 1?2 (Col 1?2, B), osteopontin (OPN, C), osteonectin (OSN, D), osteocalcin (OSC, E) and dentin sialophosphoprotein (DSPP, F), were assayed by semi-quantitative RT-PCR and each marker gene was normalized against GAPDH. The expression of each marker gene is shown as a percentage relative to its initial expression value, which is shown as 100%. Gene expression levels were significantly greater in the presence of GDF-5 than in its absence. =4 in each group, * < 0.05.

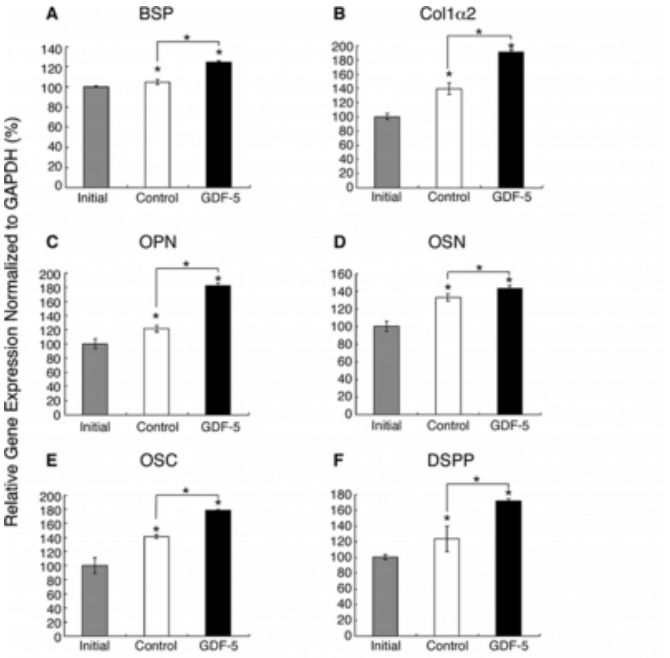

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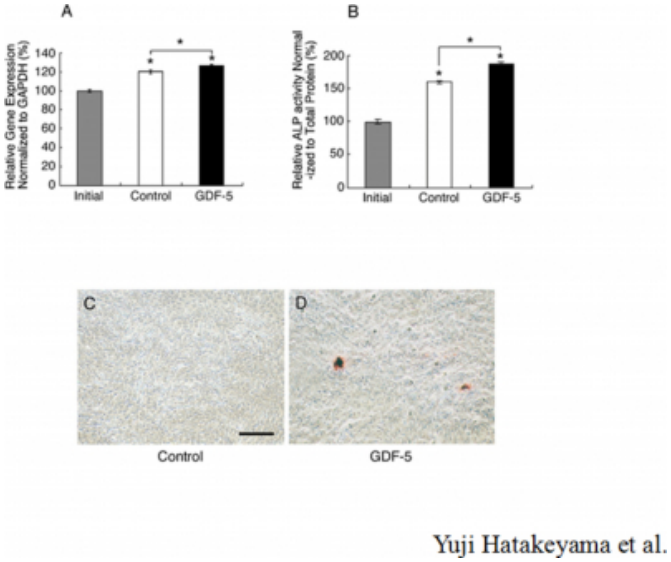
Figure 3

Fig. 3 GDF-5 promoted ALP gene expression and enzyme activity (A,B). Dental pulp cells were cultured for seven days in the absence (Control) or presence of 500 ng/ml GDF-5 (GDF-5) after cell confluence was achieved (Initial). (A) The gene expression level of ALP was assayed by semi-quantitative RT-PCR, and the expression of each gene was normalized to that of GAPDH. Gene expression levels of the Control and ALP groups are shown as percentages of their initial values, which are shown as 100% (Initial). (B) Quantitative ALP enzyme activity was determined based on light absorbance using p-nitrophenyl phosphatase as a substrate, and each ALP activity value is shown as a percentage of its initial value based on the quantity of ?mol/p-nitrophenyl phosphatase/min/?g protein. Both gene expression (A) and enzyme activity (B) of ALP was promoted significantly in the presence of GDF-5. =4 in gene expression assay, =8 in ALP activity assay. * < 0.05.



GDF-5 induced the formation of alizarin red-positive nodules (C, D). Dental pulp cells were cultured in the absence (C) or presence of 500ng/ml GDF-5 (D) and stained with alizarin red after 10 days of culture. There were no alizarin red-positive nodules in the absence of GDF-5. Bar=300□m in Fig. 3C.

DISCUSSION

GDF-5 is a member of the BMP family of proteins, a subgroup of the TGF-□ superfamily that is expressed in dental tissues including dental sacs, odontoblasts, dental pulp and periodontal ligament cells. Cells derived from dental pulp tissues are critically important as a supply of odontoblast precursor cells in the regeneration of dentin, yet the function of GDF-5 in these cells remains unknown. We showed that exogenous recombinant mouse GDF-5 increased odontoblast marker gene expression levels in cells derived from the molars of one-week-old mice after seven days of culture. All of the odontoblast marker genes that we used in this study are expressed in odontoblasts in vivo (reviewed in Butler and Ritchie, 1995), and it has been reported that dental pulp-derived cells expressing all of these marker genes formed mineralized tissues in vivo, suggesting that these cells can differentiate into functional osteo-/odontoblasts (Yang et al., 2009). We also showed that recombinant GDF-5 increased alkaline phosphatase activity in dental pulp cells and promoted the formation of alizarin red-positive nodules, suggesting that odontoblasts differentiated from dental pulp cells may form calcification nodules. Thus our results suggest that exogenous recombinant GDF-5 could promote functional odontoblast differentiation from dental pulp cells in vitro.

We assayed the effect of GDF-5 on cell proliferation and found that the presence of 500 ng/ml exogenous recombinant mouse GDF-5 had no effect on the proliferation of cells derived from dental pulp after one to seven days of culture. Several studies have likewise reported that other BMP-family proteins have no effect on the proliferation of dental pulp cells: specifically, cells derived from primary cultured human dental pulp do not exhibit increased numbers after one to seven days of culture in the presence of endogenous BMP-7 (Lin et al., 2007) or after one to 20 days of culture in the presence of exogenous recombinant BMP-2 (Saito et al., 2004). On the other hand, osteoprogenitor cells cultured with exogenous recombinant BMP-7 (Asahina et al, 1993) or BMP-2 (Yamaguchi et al, 1991) did exhibit increased cell numbers. Interestingly, we previously reported that GDF-5 had no effect on osteoprogenitor cell proliferation (Hatakeyama et al., 2011). These findings indicate that the effect of GDF-5 on cell proliferation may depend on the tissue source or origin of the cells being studied, or, more precisely, on whether the cells are involved in forming hard tissues. Indeed, in cells derived from periodontal tissue, GDF-5 at dosages ranging from 10 to 1000 ng/ml increased total cell numbers after 10 days of culture, and a significant difference appeared after eight days of culture at 1000 ng/ml (Nakamura et al., 2003). Furthermore, cells derived from mouse calvariae, consisting of a heterogeneous mixed-cell population including osteoblasts, periosteum cells and connective tissue fibroblasts, showed increased cell numbers after being cultured with recombinant GDF-5 (Yoshimoto et al., 2006). A recent study has reported that culturing dental papilla-derived cells in the presence of either recombinant

mouse GDF-5 at concentrations ranging from 10 to 1000 ng/ml or BMP-2 for five days did not result in a significant difference in total cell number (Sumita et al., 2010). Therefore our results suggest that GDF-5 has no effect on the proliferation of cells derived from molar dental pulp tissue of one-week-old mice. It has been shown that GDF-5 binds to type I receptors for BMPR-IB (also called activin receptor-like kinase (ALK)-6), as well as type II receptors for BMP, BMPR-II and activin, specifically, to activin receptor-II (ActR-II) in a rat osteoprogenitor-like cell line (Nishitoh et al., 1996). These receptors are expressed in dental pulp cells (Gu et al., 1996, Toyono et al., 1997a, 1997b) and the alternative expression of these receptors during odontoblast differentiation in vivo suggests that GDF-5 and its receptors could play an alternative role in odontoblast differentiation, depending on the cell differentiation stage, through a signaling pathway via receptors of GDF-5 (Toyono et al., 1997b). It is still unclear, however, how GDF-5 is involved in dental pulp cell proliferation; further investigations are needed to clarify the role of GDF-5 and its receptors in dental pulp cell proliferation in each stage of dental pulp tissue development.

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Author Information

Yuji Hatakeyama
Department of Morphological Biology, Functional Structure Section, Fukuoka Dental College

Yuriko Maruya
Division of Pediatric Dentistry, Orthodontics and Dentofacial Orthopedics, Craniofacial Development and Regeneration, Tohoku University Graduate School of Dentistry

Junko Hatakeyama
Department of Oral Anatomy and Cell Biology, Graduate School of Dental Science, Kyushu University

Kyoko Oka
Department of Oral Growth and Development, Pediatric Dentistry Section, Fukuoka Dental College

Eichi Tsuruga
Department of Morphological Biology, Functional Structure Section, Fukuoka Dental College

Tetsuichiro Inai
Department of Morphological Biology, Functional Structure Section, Fukuoka Dental College

Yoshihiko Sawa
Department of Morphological Biology, Functional Structure Section, Fukuoka Dental College