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ORIGINAL ARTICLE

Effect of GDF-5 and BMP-2 on the expression of tendo/ligamentogenesis-related markers in human PDL-derived cells

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OBJECTIVES: The effect of growth differentiation factor 5 and bone morphogenetic protein 2 on human periodontal ligament-derived cells was investigated with special reference to tendo/ligamentogenesis-related markers.

MATERIALS AND METHODS: Effects of each factor were analyzed by quantitative PCR for scleraxis and tenomodulin and by western blotting for scleraxis. After exposure to those factors, STRO-1-positive and STRO-1-negative fractions of human periodontal ligament tissues were isolated with an immunomagnetic cell sorting system, and the expression of scleraxis in each fraction was analyzed by western blotting. Non-separated crude cells were used as a control.

RESULTS: Growth differentiation factor 5 and bone morphogenetic protein 2 did not increase alkaline phosphatase activity in crude periodontal ligament-derived cells. Growth differentiation factor 5, but not bone morphogenetic protein 2, increased the expression of scleraxis in crude, STRO-1-positive and STRO-1-negative periodontal ligament-derived cells. The expression of scleraxis in STRO-1-positive periodontal ligament-derived cells was significantly less compared to that in crude P2 and STRO-1-negative periodontal ligament-derived cells.

CONCLUSION: Growth differentiation factor 5 induced the expression of scleraxis and may enhance tendo/ligamentogenesis in human periodontal ligament-derived cells. The expression of scleraxis was higher in STRO-1-negative fraction, suggesting more differentiated state of the cells.

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Introduction

Periodontal ligament (PDL) is anticipated as a suitable material for periodontal regeneration. But the mechanism of periodontal regeneration is not well known. Recent findings suggest the presence of somatic stem cells in PDL tissue, which are defined as STRO-1 expression (Seo *et al*, 2004). Therefore, it is considered that the STRO-1-positive (STRO-1^{+}) putative stem cells may proliferate and differentiate into both periodontal cells and cementoblasts to regenerate periodontal tissue when it is damaged.

Growth differentiation factor 5 (GDF-5), also known as cartilage-derived morphogenetic protein-1, is a member of the bone morphogenetic protein (BMP) family and the transforming growth factor-β (TGF-β) superfamily. GDF-5 is known as a key regulatory factor of limb skeletal development (Storm *et al*, 1994; Chan *et al*, 1994; Thomas *et al* 1996; Merino *et al*, 1999; Storm and Kingsley, 1999) as well as a regulatory gene of bone, cartilage, and tendon/ligament formation. Although it is known that GDF-5 localizes in periodontal tissue, its physiological roles under normal and diseased conditions remain to be investigated. Our group has demonstrated that GDF-5 regulates differentiation of both dental papilla and follicle during odontogenesis and may co-operatively play crucial roles with other growth factors such as BMP-2 in porcine cells (Sumita *et al*, 2010). Recently, the potential of GDF-5 to regenerate periodontal tissue has been investigated using animal models (Kim *et al*, 2009; Kwon *et al*, 2010;

Lee *et al*, 2010), and the effect of GDF-5 on osteogenic differentiation of human PDL-derived cells was also reported (Nakamura *et al*, 2003). However, available information about its effect on tendo/ligamentogenesis-related markers is still limited. As GDF-5 regulates not only bone and cartilage formation but also tendo/ligamentogenesis during development, it is conceivable to speculate the importance of the role of GDF-5 in the regulation of ligament-related markers in PDL-derived cells.

Bone morphogenetic proteins were originally identified as a molecule to induce ectopic bone formation (Urist, 1965). BMPs also play a variety of roles during development and cell differentiation (Valenzuela *et al*, 1995; Jamali *et al*, 2001; Warren *et al*, 2003). Among the BMPs, BMP-2 is well known as a strong osteogenic inducer and has been clinically used in cases of spinal fusion and alveolar bone regeneration (Kwong and Harris, 2008). Efficacy of recombinant human BMP-2 (rhBMP-2) for periodontal tissue repair has been investigated in animal models. Kinoshita *et al* (1997) reported that rhBMP-2 improved new bone formation, new cementum formation, and connective tissue attachment in circumferential defects created by experimental periodontitis in beagle dogs, and similar findings were also reported using a rat model (King *et al*, 1997). On the other hand, some recent studies have shown the limited effect on cementum formation (Choi *et al*, 2002) and increased ankylosis at the treated sites (Wikesjo *et al*, 2003; Takahashi *et al*, 2005). Currently, the efficacy of BMP-2 on periodontal tissue regeneration remains controversial. Previous studies investigated the effect of BMP-2 on PDL-derived cells and osteoinductive but not proliferation-inducing effects were reported (Kobayashi *et al*, 1999; Zaman *et al*, 1999). However, the effect of BMP-2 on the expression of tendo/ligamentogenesis-related markers has not been reported. Furthermore, those studies used only crude PDL-derived cells, and the effect on isolated putative stem cells was not known.

There are few reports concerning the tendo/ligamentogenesis effect of growth factors on PDL-derived cells. In this study, the effect of BMP-2 and GDF-5 on crude human PDL-derived cells was investigated with special reference to tendo/ligamentogenesis-related markers to regenerate PDL, tendon, or ligament using PDL-derived cells. Furthermore, the effect of those growth factors on either STRO-1^{+} putative stem cells or STRO-1^{-} cells was also investigated and compared with crude cells.

Materials and methods

Tissue preparation and cell culture

Periodontal ligament tissues were harvested from extracted teeth (n = 10; average age = 25.8 ± 2.5) of impacted, healthy third molars. The procedure used to harvest the extracted teeth from humans conformed to the tenets of the Declaration of Helsinki, and the experimental protocol was approved by the Ethical Committee at Nagoya University School of Medicine. Informed consent was obtained from each subject prior to donation of the tissue. Each tooth samples was rinsed twice in a phosphate-buffered saline (PBS) solution containing 1000 units ml^{-1} penicillin G sodium, 1 mg ml^{-1} streptomycin sulfate, and 2.5 μg ml^{-1} amphotericin B (Invitrogen, Carlsbad, CA, USA) for 5 min at room temperature. PDL tissue was mechanically removed from the root surface by a scalpel. To avoid contamination by gingival and apical tissue, tissue from the apical and coronal regions of the tooth was discarded. Tissue was digested with 2 mg ml^{-1} collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 1 h in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Thermo Trace Ltd, Melbourne, Australia) and antibiotic–antimycotic solution on a shaker at 37°C. The cell suspension was then centrifuged for 5 min at 440 g, and the cell pellet was re-suspended in culture medium (DMEM containing 10% FBS and antibiotic–antimycotic solution). Isolated cells were seeded in a 12-well culture dish. When cells reached 80–90% confluence, they were subcultured (1 × 10^{4} cells in a 10 cm dish) until passage 2.

Treatments with osteogenic induction medium and culture medium with GDF-5 or BMP-2

Osteogenic induction medium consisted of culture medium with 10 nM dexamethasone (Sigma-Aldrich), 100 μM ascorbic acid (Wako, Tokyo, Japan), and 10 mM glycerol 2-phosphate disodium salt hydrate (β-glycerophosphate, Sigma-Aldrich). The optimal concentrations for recombinant mouse GDF-5 (rmGDF-5; R&D Systems, Inc., Minneapolis, MN, USA) and recombinant human BMP-2 (rhBMP-2; R&D Systems, Inc.) treatments were 200 and 100 ng ml^{-1} respectively, based on our preliminary study. First passage PDL-derived cells were seeded at a concentration of 5 × 10^{4} cells per well into 6-well plates. When the cultured cells reached 70% confluence, the cells were subcultured in culture medium (control group), osteogenic induction medium (Dex group), or culture medium with rmGDF-5 (200 ng ml^{-1}, GDF-5 group) or rhBMP-2 (100 ng ml^{-1}, BMP-2 group).

Total cell number and alkaline phosphatase (ALP) activity analyses

Cell proliferation and ALP activity were evaluated at 7 days among control, Dex, BMP-2, and GDF-5 groups. Cell proliferation was measured using WST-8 kit (Wako) according to the manufacturer's protocol. The quantity of pigments was then determined spectrophotometrically. Cells were incubated with medium containing 100 μl ml^{-1} of WST-8 for 1 h. The absorbance was then measured on a spectrophotometer at 450 nm (SmartSpeck™ 3000; BIO-RAD, Tokyo, Japan). ALP activities were measured according to the method of Lowry (1955). An aliquot (5 μl) of the supernatant was added to 1 ml of 50 mM p-nitrophenylphosphate containing 1 mM $MgCl_2$ (Sigma-Aldrich) and the mixture were incubated for 6 min at 37°C. 1 ml of 0.2 N NaOH was added to stop the enzymatic reaction, and the absorbance was read at 415 nm with a

spectrophotometer (SmartSpeck™ 3000; BIO-RAD). Each experiment was performed in triplicate for each of three samples.

Reverse transcription-polymerase chain reaction and quantitative PCR
Quantitative PCR (q-PCR) was used to determine the expression of tendo/ligamentogenesis-related genes in human PDL-derived cells cultured with or without rmGDF-5 (200 ng ml^{-1}) and rhBMP-2 (100 ng ml^{-1}) at 1, 3, and 7 days. Total cellular RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis was performed on 2 μg of total RNA by Super Script First-strand Synthesis (Invitrogen) according to the manufacturer's protocol.

Samples were incubated in a Thermal Cycler GP (Takara Bio Inc.) at 95°C/(2 min) for 1 cycle and then 95°C/(60 s), 56°C/(60 s), and 72°C/(60 s) for 20 cycles, with a final 5 min extension at 72°C. After amplification, 10 μl of each product was analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Furthermore, the effect of rmGDF-5 and rhBMP-2 on the expression of scleraxis and tenomodulin (TeM) mRNA in cultured PDL-derived cells was also evaluated by real-time fluorescent quantitative PCR using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster, CA, USA). Reactions were carried out with SYBR Green Master Mix (Applied Biosystems) according to manufacturer's protocol. The PCR consisted of an initial enzyme activation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. PDL-derived cell-specific primer sets used in this experiment were as follows:

Scleraxis
(forward 5′-TGCGAATCGCTGTCTTTC-3′,
reverse 5′-GAGAACACCCAGCCCAAA-3′) (91 bp),

TeM
(forward 5′-TTGAAGACCCACGAAGTAGA-3′,
reverse 5′-ATGACATGGAGCACACTTTC-3′) (119 bp),

GAPDH
(forward 5′-GCACCGTCAAGGCTGAGAAC-3′,
reverse 5′-ATGGTGGTGAAGACGCCAGT-3′) (106 bp).

Magnetic activated cell sorting (MACS)
At passage 2, PDL were subjected to immunomagnetic cell sorting using a mini-MACS isolation kit (Miltenyi Biotech, Inc, Bergisch Gladbach, Germany) according to the manufacturer's recommendations. PDL were incubated for 60 min at 4°C with monoclonal anti-human STRO-1 antibody (R&D Systems, Inc.). After incubation, cells were rinsed three times in PBS. Subsequently, the cells were incubated with rat anti-mouse IgM microbeads. After washing with PBS, the labeled cells were filtered through a 70-μm nylon mesh and loaded onto a column surrounded by a magnetic field. STRO-1-positive (STRO-1^{+}) cells bound to the STRO-1 microbeads were trapped in the column. Both the STRO-1^{+} and STRO-1-negative (STRO-1^{-}) fractions of PDL were collected for western blot analysis.

Western blot analysis
To evaluate the effect of GDF-5 on tendo/ligamentogenesis of PDL-derived cells, western blotting was performed for scleraxis protein. Crude P2 PDL-derived cells were cultured on a 10-cm culture dish until 70% confluent, and washed with PBS twice. They were then cultured for 7 days in serum-free medium with rmGDF-5 (GDF-5-treated group) or without rmGDF-5 (non-treated group), and collected for western blot analysis.

To evaluate scleraxis expression pattern, 7-day-rmGDF-5-treated P2 PDL-derived cells were sorted using MACS, and STRO-1-positive and STRO-1-negative fraction of PDL-derived cells were collected, respectively, for western blot analysis. Cells were homogenized in protein extraction buffer (CytoBuster™; Novagen, Merck KGaA, Darmstadt, Germany) with protease inhibitor cocktail tablets (Complete Mini; Roche, Indianapolis, IN, USA). The protein concentration for the lysate was measured by BCA assay kit (Pierce, Rockford, IL, USA), and the protein was denatured by boiling with SDS and 2-mercaptoethanol solution. An equal concentration of proteins in 7.5–12.5% SDS-polyacrylamide gel was applied to samples to perform electrophoresis. The protein was transferred by iBlot TM Dry Blotting System (Invitrogen). The blotting membrane (nitrocellulose) was blocked with 4% skim milk in PBS at room temperature for 60 min, then immunoblotted using a rabbit polyclonal antibody to scleraxis (abcam; 1:500). Secondary anti-rabbit antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) at a 1:3000 dilution were used for the detection and visualized using a chemoluminescence ECL detection system (Amersham Life Sciences, Arlington Heights, IL, USA). To assess the intensity of bands for these proteins quantitatively, densitometric analysis was performed using ChemiDoc (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with imaging software (Quantity One; Bio-Rad). The intensity level of detected protein bands was divided by the intensity level of β-actin to be standardized. The relative scleraxis expression was defined as the level of expression in each sample divided by that of non-GDF-5-treated cells from the same donor (Figure 3c,d) and crude PDL-derived cells from the same donor (Figure 4b).

Statistical analysis
One-way repeated measures ANOVA were used for western blot analyses to detect any significant difference within each group. When a significant difference was detected, the difference among any selected groups was confirmed using Dunnett's test. Experimental values are presented as mean ± s.d. A P-value of < 0.05 was considered to be statistically significant.

Results

Osteogenic differentiation of PDL-derived cells
When crude PDL-derived cells at second passage were treated by osteogenic induction medium, ALP activity increased significantly compared to the other groups. On the other hand, the levels of ALP activity did not change when the cells were treated with either rmGDF-5 or rhBMP-2 (Figure 1).

Effect of GDF-5 and BMP-2 on the expression of tendo/ligamentogenesis-related genes in crude P2 PDL-derived cells
The results from q-PCR showed that rmGDF-5 tended to upregulate the expression of *scleraxis*, although the difference was not significant possibly due to the large variation (Figure 2a). A similar tendency was observed when the expression of *TeM* was analyzed. rmGDF-5 tended to upregulate the expression of *TeM* at all time points and rhBMP-2 tended to upregulate the expression of *TeM* only at day 7, although the differences were not significant (Figure 2b).

Effect of GDF-5 on the expression of scleraxis protein in crude P2 PDL-derived cells and STRO-1⁻ fraction
The results from western blot analysis showed that both crude P2 PDL-derived cells and STRO-1$^-$ fraction of PDL-derived cells expressed scleraxis protein (Figure 3a,b). There was a significant difference among GDF-5-treated and non-treated crude P2 PDL-derived cells (1.86 ± 0.77, $P < 0.05$) (Figure 3c). In terms of STRO-1$^-$ fraction of PDL, similar tendency was observed, although the difference was not significant (Figure 3d).

Expression pattern of scleraxis protein in GDF-5-treated crude, STRO-1$^+$ and STRO-1$^-$ P2 PDL-derived cells
The results from western blot analysis showed that crude P2, STRO-1$^+$, and STRO-1$^-$ PDL are treated with GDF-5 expressed scleraxis protein (Figure 4a). The relative expression level of scleraxis in STRO-1$^+$ PDL-derived cells was significantly smaller than that in crude cells (0.63 ± 0.17, $P < 0.05$). On the other hand, the relative expression of scleraxis in STRO-1$^-$ PDL-derived cells was almost identical to that in crude cells (0.91 ± 0.10) and significantly higher than that in STRO-1$^+$ PDL-derived cells ($P < 0.05$) (Figure 4b).

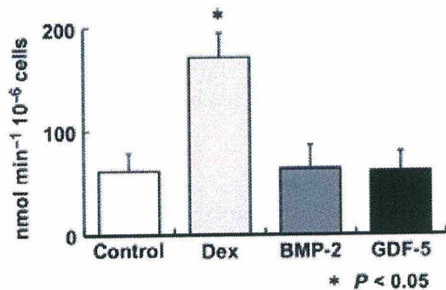

Figure 1 Alkaline phosphatase (ALP) activity of crude PDL-derived cells in passage 2. ALP activity of the Dex group was significantly greater compared with control, BMP-2, and GDF-5 groups. *$P < 0.05$. Values are the mean ± s.d. of five experiments

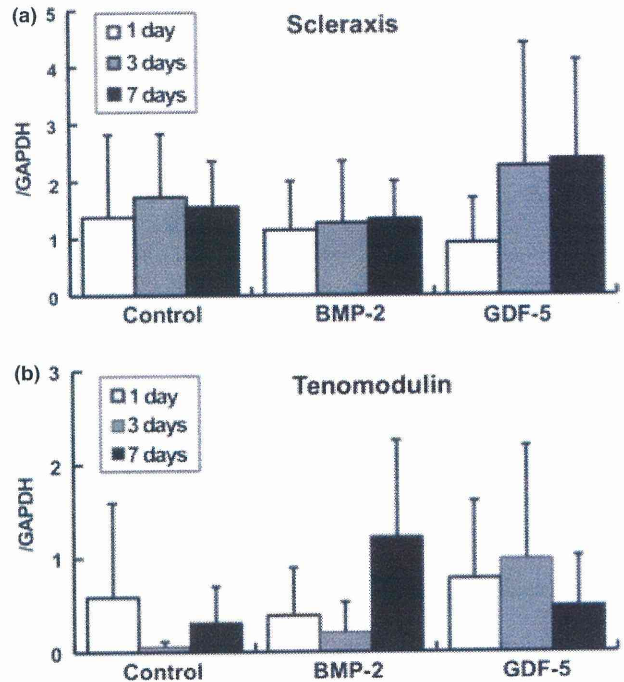

Figure 2 Quantitative RT-PCR analysis for scleraxis (**a**) and tenomodulin (**b**) gene expression of crude PDL-derived cells in passage 2. Cells were cultured with culture medium with or without BMP-2, GDF-5 for 1, 3, and 7 days. There were no significant differences among control, BMP-2, and GDF-5 groups on any time points. Values are the mean ± s.d. of five experiments

Discussion

PDL-derived cells constantly express both osteogenic/cementogenesis- and tendo/ligamentogenesis-related genes. Although the results from a study using clonal cells demonstrate that single colony-derived PDL cells can express both osteogenic/cementogenesis- and tendo/ligamentogenesis-related genes, crude PDL-derived cells generally consist of heterogeneous multiple colonies (Itaya *et al*, 2009), and the physiology of crude PDL cells may not be identical to that of putative stem cells. A recent study reported that STRO-1 is considered as a marker for mesenchymal stem cells including human PDL stem cells (Seo *et al*, 2004). Although the ratio of STRO-1$^+$ cells in PDL cells varies among research reports, our previous study demonstrated a 33.5% rate of STRO-1$^+$ cells in primary culture, which dropped to 14.7% by the third passage (Itaya *et al*).

The results from osteogenic induction with dexamethasone confirmed that crude PDL-derived cells showed increased ALP activities. However, rmGDF-5 and rhBMP-2 did not affect the ALP activity, which suggest that those factors may not induce bone formation but allow retaining characteristics of ligament cells at the concentration used in this study. Although the results

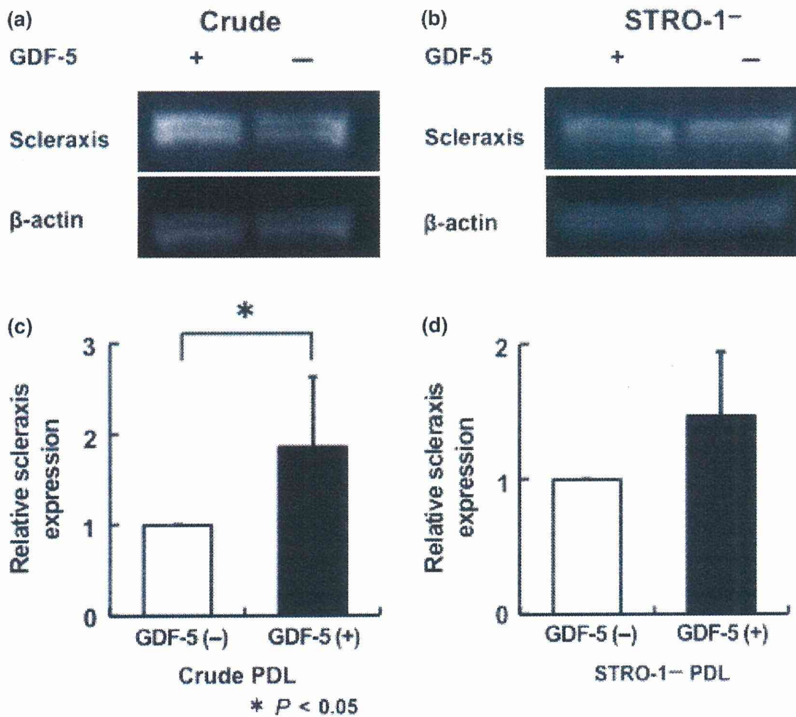

Figure 3 Western blot analyses of scleraxis crude PDL-derived cells (**a**) and STRO-1^{-} PDL-derived cells (**b**) at passage 2. Expression of scleraxis was detected in all samples. An experiment representative of five similar studies is shown. (**c**) GDF-5-treated crude PDL-derived cells had significantly higher scleraxis expression than the other groups. (**d**) There was a similar tendency to crude PD-L-derived cells, but no significant difference in STRO-1^{-} PDL-derived cells among all groups. Values are the mean ± s.d. of five experiments

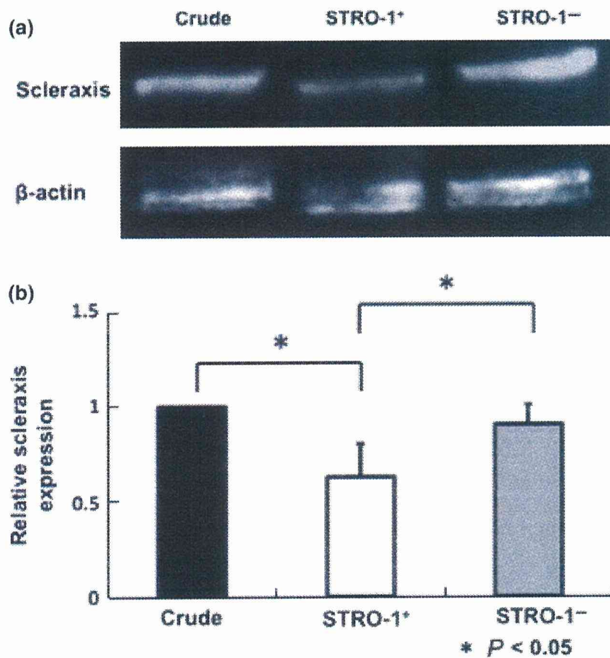

Figure 4 The results from western blot analyses of scleraxis in crude PDL-derived cells, STRO-1^{+}, and STRO-1^{-} PDL-derived cells at passage 2. All samples were treated with rmGDF-5. (**a**) The expression of scleraxis proteins was detected in all groups for 7 days. An experiment representative of six similar studies is shown. (**b**) Reduced scleraxis expression in STRO-1^{+} PDL-derived cells was statistically significant compared to crude P2 and STRO-1^{-} PDL-derived cells. Values are the mean ± s.d. of six experiments

from this study are based on a single dose of rmGDF-5, GDF-5 is known to decrease ALP activity of human PDL-derived cells dose dependently, and the results from this study may be consistent with the previous findings (Nakamura *et al*, 2003). BMP-2 is a strong inducer of osteogenic differentiation in various types of cells. In terms of human PDL-derived cells, BMP-2 may require preconditioning osteogenic induction medium with dexamethasone to induce ALP activities (Hou *et al*, 2007). The result from this study demonstrated that BMP-2 alone was not effective in inducing osteogenic differentiation of crude human PDL-derived cells.

In this study, we focused on the effect of GDF-5 and BMP-2 on the expression of tendo/ligamentogenesis-related genes that is *scleraxis*, *TeM*. Scleraxis is a helix-loop-helix transcription factor that has been shown to be a highly specific marker for tendon and ligament progenitors and differentiating cells (Cserjesi *et al*, 1995; Schweitzer *et al*, 2001; Brent *et al*, 2003, Banos *et al*, 2008). It has been suggested that scleraxis is required for normal tendon differentiation and formation, presumably via regulation of collagen type I promotion (Banos *et al*, 2008). The presence of scleraxis in PDL-derived cells has been reported (Shi *et al*, 2005; Itaya *et al*, 2009). TeM is a member of a new family of type II transmembrane glycoproteins and is predominantly expressed in tendon, ligaments, and eyes (Docheva *et al*, 2005). TeM is known as a late marker of tendon formation, and the presence of TeM in human PDL has been reported (Itaya *et al*, 2009). The results from this study showed that both GDF-5 and BMP-2 affect the differentiation of PDL-derived cells *in vitro*. However, the effect on those differential ligament-makers was not identical and the underlying mechanisms might be complex.

In terms of GDF-5, the induction of scleraxis was demonstrated in crude PDL-derived cells. This finding suggests the potential usefulness of this factor for PDL

regeneration. On the other hand, scleraxis expression was significantly less in GDF-5-treated STRO-1^+ fraction. One possibility is that STRO-1^+ PDL-derived cells are putative stem cells and not all cells were committed into osteogenic or tendon/ligament lineage yet. Interestingly, the sum of scleraxis expression in STRO-1^- and STRO-1^+ PDL-derived cells was less than that of crude cells. The reason for this phenomenon is not clear from this study but it may suggest the important role of cell-to-cell interaction in the maintenance of differentiated status. Cell-to-cell interaction can be achieved via cytokines, growth factors, and also direct contact. Recently, a novel mechanism of cell-to-cell interaction via microvesicles has been reported. Accumulating evidence has shown that stem cells can interact with adjacent and even distant cells with microvesicles, which can transport proteins as well as RNA and affect other cells (reviewed by Camussi *et al*, 2010). Further analyses are required to understand the mechanisms for this differential effect.

The effects of BMP-2 on tendo/ligamentogenesis-related gene expression of PDL-derived cells were heterogeneous. As excessive bone formation around root surface induces ankylosis, the maintenance of PDL is essential for the successful regeneration of periodontal tissue. The usefulness of BMP-2 on periodontal tissue regeneration should be discussed regarding both osteogenesis/cementogenesis and ligamentogenesis around the root surface, which may require further justification.

This study focused on the tendo/ligamentogenesis-related markers and confirmed the effect of those factors not only on crude PDL-derived cells but also on STRO-1^+ and STRO-1^- PDL-derived cells. The results from our study showed some potential beneficial effect of GDF-5 on periodontal tissue regeneration. However, the underlying mechanisms appear to be complicated, and the overall benefit of the clinical application of the factor requires further analyses.

Author contributions

Minoru Inoue involved in acquisition, analysis of data; Katsumi Ebisawa involved in research design, drafting & revising the paper; Toshimitsu Itaya and Takayuki Sugito involved in interpretation of data; Aika Yamawaki-Ogata involved in acquisition, analysis of data; Yoshinori Sumita involved in interpretation of data; Ryu Wadagaki involved in acquisition of samples; Yuji Narita involved in research design; Hideki Agata involved in interpretation of data; Hideaki Kagami involved in research design, drafting the paper involved in Minoru Ueda involved in research design.

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Ischemic culture of dental pulp-derived cells is a useful model in which to investigate mechanisms of post-ischemic tissue recovery

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Summary

Dental pulp is a soft tissue characterized by unique regenerative properties. It is located in the center of each tooth, and is surrounded by hard tissue (dentin). Vascular access is limited to a small foramen at the root apex. Because of this anatomical limitation, dental pulp can easily lose its blood supply, causing the tissue to become ischemic. This occurs, for example, when a tooth is dislocated by traumatic injury or is subjected to inflammation. Since ischemia is caused by a critical shortage of oxygen and nutrients, ischemic damage is usually irreversible, even when the ischemic event is transient. However, unlike ischemia-sensitive organs such as the brain and heart, dental pulp is relatively ischemia-resistant, and recovers from ischemic injury by regenerating damaged tissue. The mechanisms by which this regeneration occurs are poorly understood, but are being investigated in cell culture models that mimic *in vivo* ischemic conditions using a combination of hypoxia and nutrient deprivation. Here, we review the use of ischemic cell culture to investigate the mechanisms of post-ischemic dental pulp tissue recovery.

Introduction

Ischemia occurs when arterial blood flow to a tissue is restricted. Because arterial blood supplies oxygen and nutrients, ischemia is characterized by a lack of these essential elements (Shinzawa and Tsujimoto, 2003). The intensity of tissue damage depends on the length and severity of the ischemic event. Although direct comparison is difficult, tolerance to ischemia appears to differ among various tissues and organs. For example, brain tissue is highly vulnerable to ischemia, and often becomes necrotic even when only transiently exposed to ischemic conditions (Sugiyama et al., 2011). In contrast, dental pulp is relatively tolerant to ischemia and is able to survive transient ischemic events such as tooth extraction and replantation (Tsukamoto-Tanaka et al., 2006). Dental pulp recovers its functions by regenerating damaged tissue after ischemia exposure. This unique response suggests that dental pulp contains ischemia-tolerant cells that play important roles in post-ischemic tissue regeneration.

Dental pulp contains a postnatal stem cell population named dental pulp stem cells (DPSCs) (Gronthos et al., 2000). DPSCs possess multi-lineage differentiation abilities (odontogenic, osteogenic, chondrogenic, adipogenic and neurogenic lineage), and are considered potent stem cells for use in tissue engineering and regenerative medicine (Gronthos et al., 2002, Iohara et al., 2006).Although the characteristics and functions of

DPSCs within the pulp remain largely unknown, these cells appear to play an important role in tissue development, homeostasis, and regeneration and are a particularly interesting target for investigations into the mechanisms of post-ischemic tissue regeneration.

Evaluating dental pulp tissue reactions during and after ischemia would ideally be undertaken *in vivo*, but this is experimentally difficult due to the anatomic location of the tissue and its complex cellular composition (Liu et al., 2006). Thus, *in vitro* ischemic culture of dental pulp-derived cells has been developed as an alternative experimental model, though studies differ in the culture conditions utilized (Agata et al., 2008, Wang et al., 2010). In this review, we discuss *in vitro* cell culture conditions that best approximate *in vivo* ischemia. Next, we evaluate the relevance of these conditions in dental pulp-derived cell culture. Finally, we examine the characteristics of dental pulp-derived cells that survive ischemic culture conditions and explore possible mechanisms of post-ischemic pulp tissue regeneration.

Approximating *in vivo* ischemic conditions in experimental cell culture systems

Under ischemic conditions, cells experience both low oxygen tension and nutrient deprivation. Hence, ischemia can be mimicked *in vitro* by exposing cells to both hypoxia

and a low-glucose environment (Jones et al., 2011). These appear to be the two most influential factors for tissue survival (Acosta et al., 1978). When PC12 cells (derived from a pheochromocytoma and able to differentiate into neurons) are cultured under hypoxic conditions and in a low-glucose environment, they are severely damaged, often to the point of necrosis (Shinzawa and Tsujimoto, 2003). Unfortunately, most of the current literature on ischemic culture of dental pulp-derived cell or DPSCs is limited to investigation of the effect of low oxygen tension alone; the number of studies using both low oxygen tension and nutrient deprivation is limited (Agata et al., 2008; Wang et al., 2010) (Table 1). In fact, low nutrient supply may enhance the effect of low oxygen tension. It has been shown that caspase-independent cell death, which is commonly seen under ischemic conditions, is significantly upregulated when cells are deprived of both oxygen and glucose (Agata et al., 2008).

Another important consideration when developing *in vitro* models of ischemia is the level of hypoxia used in the experiments. Conventional cell culture experiments use approximately 20% oxygen, with a partial pressure of oxygen (pO_2) of 140 mmHg (Rodrigues et al., 2010). However, pO_2 in the arterial blood of normal human subjects ranges from 60-90 mmHg and pO_2 in bone marrow is even lower (47-49 mmHg). The discrepancy between *in vitro* and *in vivo* conditions suggests that conventional cell

culture may occur in a relatively hyperoxic environment, while traditional "hypoxic" culture environments actually reproduce normal physiologic conditions. This may explain why mesechymal stem cells grow and survive better in low oxygen cell culture environments. Mesenchymal stem cells cultured under low oxygen tension (5%) have a greater number of colonies as primary isolates, proliferate more rapidly and produce more bone (Lennon et al., 2001). In fact, "hypoxic" cell culture conditions can increase proliferation rates and enhance differentiation along multiple mesenchymal lineages (Das et al., 2010), providing further evidence that the oxygen levels in traditional hypoxic cell culture experiments may not accurately reflect *in vitro* ischemic environments. The oxygen tension within dental pulp tissue *in vivo* is difficult to measure directly. However, it is possible that the oxygen range commonly used in "hypoxic" cell culture experiments (2-5%) may not be an accurate approximation of *in vivo* conditions, and very low oxygen tension (less than 1%) might be necessary (Agata et al., 2008).

Experimental ischemia in dental pulp-derived cell culture

Several studies have demonstrated that low oxygen tension (2-5%) promotes proliferation of dental pulp-derived cells (Amemiya et al., 2003; Sakdee et al., 2007; Iida et al., 2010; Li et al., 2011). This finding may reflect the fact that dental pulp-derived cells

are exposed to relatively low oxygen tension within their normal physiologic environment. When these cells are exposed to both hypoxia (2%) and serum deprivation for 24 or 48 hours, a condition that mimics *in vivo* ischemia, proliferation rates decline (Wang et al., 2010). Cells survive in this environment even though proliferation rates decline, suggesting a complex cellular response to ischemia. This response includes suppression of cell growth and induction of cellular defense systems, including upregulation of hypoxia-inducible factor 1α (HIF-1α), heat shock protein 70 (HSP 70), and AMP-activated protein kinase (AMPK) (Amemiya et al., 2003; Fukuyama et al., 2007; Agata et al., 2008; Aranha et al., 2010). These factors may even activate cell growth after the ischemic event has resolved (Ueno et al., 2006; Fukuyama et al., 2007). This complex response to hypoxia and nutrient deprivation reflects a balance between damage due to noxious stimuli and activation of cellular defense systems.

The effect of low oxygen tension on the differentiation capacity of dental pulp-derived cells is unclear. After being cultured for 14 days in 5% O_2, dental pulp-derived cells increase expression of osteonectin (ON), dentin matrix protein-1 (DMP-1), bone sialoprotein (BSP) and dentin sialophosphoprotein (DSPP), and after 21 days significantly greater calcified nodule formation is observed. Both of these factors appear to promote differentiation (Li et al., 2011). Other studies have reported that

hypoxia has an inhibitory effect on differentiation of dental pulp-derived cells. For example, canine dental pulp-derived cells show decreased alkaline phosphatase activity (ALP, an early marker of osteogenic (odontogenic) differentiation) after 4 days in both hypoxic (2% O_2) and normoxic culture conditions, though ALP activity in the hypoxic cells remained higher than in the normoxic cells (Amemiya et al., 2004). Further, we have demonstrated that porcine dental pulp-derived cells have significantly lower expression of BSP, DMP-1, and DSPP in both differentiation-induced and non-induced cells regardless of the severity of ischemia , though expression of other marker genes is not significantly different when cells are grown in glucose-containing medium (Fig. 1). Additionally, when human dental pulp cells are cultured under 3% O_2 for 14 days, ALP activity and the expression of DMP1, DSPP and osteocalcin are suppressed (Iida et al., 2010).

Investigations into the effect of low oxygen tension on the ability of dental pulp-derived cells to differentiate differ in terms of the species from which the cells were isolated and the conditions under which the cells were cultured (i.e. level and duration of hypoxia, and the status of cell differentiation), making direct comparisons difficult. However, accumulating evidence indicates that ischemic conditions do affect the differentiation of dental pulp-derived cells, and the type and magnitude of the effect may

correlate with the severity of ischemia. Detailed cellular analyses are required to understand the mechanisms underlying this phenomenon.

Characteristics of dental pulp-derived cells that survive under ischemic cell culture conditions.

Dental pulp tissue often recovers its function after an ischemic event, and ischemia-surviving cells likely contribute to tissue recovery. As previously discussed, traditional low oxygen culture conditions may not accurately reflect hypoxic environments *in vivo*. Accordingly, very low oxygen tension (<0.1% O_2, or even anoxia) is required for culture conditions to truly approximate physiologic environments (Fig. 2) (Agata et al., 2008). Furthermore, these cultures may require "ischemic conditions", which involves not only very low oxygen tension but also nutrient deprivation (Agata et al., 2008; Wang et al., 2010).

Investigations into the mechanisms by which dental pulp-derived cells recover from ischemia have focused on determining whether cellular differentiation affects survival. However, the number of experiments performed with very low oxygen tension (hypoxia) or ischemic conditions (hypoxia plus nutrient deprivation) is limited. We have shown that differentiation-induced and non-induced cells (possibly stem/progenitor cells) survive at

equivalent rates under a range of ischemic conditions. Hence ischemia-tolerance is comparable between differentiated and non-differentiated cells (Agata et al., 2008). Despite these similarities, differences between the two cell populations do exist. Upregulation of the pluripotent stem cell markers octamer-binding transcription factor 4 (Oct4) and Sox2 is observed only in non-induced cells under ischemic conditions (Fig. 3). This finding suggests that ischemia-surviving non-odontogenic cells (undifferentiated cells) may be able to de-differentiate, acquiring greater growth and differentiation potential for post-ischemia tissue regeneration. In contrast, de-differentiation of ischemia-surviving odontogenic (differentiated) cells may allow them to re-acquire mitotic potential (pulp-resident odontoblasts are post-mitotic cells that are not able to divide or repair damaged dentin) (Liu et al., 2006). Hence cells that survive ischemic insult may de-differentiate under ischemic conditions and obtain more stem cell-like characteristics, thereby contributing to the regeneration of damaged tissue.

Insights into the mechanisms of dental pulp tissue recovery after ischemic damage

Cellular damage caused by ischemia is generally irreversible even when the ischemic insult is transient. Dental pulp tissue can recover its functions after transient ischemia, suggesting that this tissue may undergo cellular regeneration rather than cellular recovery.

The physiologic properties of dental pulp (protection, nutrition, and tooth sensation) are supported by multiple cell types, hence post-ischemic pulp recovery has previously been attributed to multiple cell populations (Sloan and Smith, 2007). However, with the discovery of dental pulp stem cells (DPSCs) which are able to differentiate into multiple cell lineages (angiogenic, vasculogenic, neurogenic, chondrogenic, and osteo/odontogenic) a new paradigm has emerged in which a single stem cell population may be all that is required for pulp tissue recovery. Thus, it is of interest to investigate whether the post-ischemic recovery of pulp function is mediated solely by DPSCs.

One of the most important functions of dental pulp is to protect the tooth from noxious stimuli through dentin formation. Reparative dentin formation is frequently observed in teeth that survive ischemic insults (Spahr et al., 2002). The cell types responsible for this restorative process are unknown. Dental pulp contains terminally differentiated odontoblasts, but these cells are considered post-mitotic and thus not able to divide and form dentin (Liu et al., 2006). Additionally, odontoblasts may become necrotic under ischemic conditions. This has led to the assumption that reparative dentin formation following an ischemic event might be solely mediated by DPSCs (though other functions appear to be recovered by multiple cell populations) (About and Mitsiadis, 2001, Liu et al., 2006). However, as stated above, results from ischemic culture of

induced dental pulp cells (odontoblast-like cells) indicate that resident committed odontoblasts/precursors may de-differentiate and re-acquire mitotic potential during ischemia (Fig. 1). Therefore, post-ischemic reparative dentin formation may in fact be mediated by both DPSCs and de-differentiated odontoblasts, though further investigation is required to confirm this. If pulp-resident odontoblasts do contribute to post-ischemic reparative dentin formation, their ability to form dentin may be lower than that of DPSCs, because ALP activity of de-differentiated cells is lower in induced cells than in non-induced cells (which possibly contain DPSCs) (Fig. 4A and 4B).

Investigations into the post-ischemic recovery of other physiologic functions of dental pulp tissue are rare (e.g. nutrition and tooth sensation), but studies in other tissues demonstrate reparative roles for multiple cell types, including endothelial and neural cells (Sheridan and Bonventre, 2000, Bernert et al., 2003). Thus a more complete understanding of the mechanism of post-ischemic pulp tissue recovery should include analysis of endothelial and neural cell populations residing in dental pulp tissue. Finally, angiogenic factors such as vascular endothelial growth factor (VEGF) are induced under hypoxic conditions, which may support the durability of dental pulp during hypoxia in vivo (Amemiya et al., 2003; Aranha et al., 2010).

Conclusion

It has long been known that dental pulp recovers its function after transient ischemia, but the mechanisms underlying this phenomenon have not been fully investigated, in part because *in vivo* monitoring of this tissue is anatomically difficult. Cell culture is emerging as a useful model for examination of cellular responses to ischemia and may become a valuable approach for investigation into the mechanisms of post-ischemic pulp tissue recovery.

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