Expression of Odontoblastic-Related Genes in Human Dental Follicle Cells, Dental Pulp Stem Cells, and Oral Mucosal Cells

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Abstract

In the current studies, we examined the ability of human dental follicle cells (DFCs), dental pulp stem cells (DPSCs), and oral mucosal cells (OMCs) to form dentin by analyzing their expression of the odontoblast-specific genes, dentin sialophosphoprotein (DSPP) and dentin sialoprotein (DSP), and the mineralization-associated genes, alkaline phosphatase (ALP) and dentin matrix protein-1 (DMP-1). Phase contrast microscopy showed that DFCs, DPSCs, and OMCs exhibited spindle-shaped and fibroblastic morphologies. Also, more bone nodules were formed in DFCs than in DPSCs, whereas bone nodules were not found in OMCs. Analysis of gene expression by real-time reverse transcriptase-polymerase chain reaction showed a time-dependent increase in the expression of DSPP in differentiating DFCs and DPSCs, but almost no expression in OMCs. The expression of DSPP in DFCs was approximately 4-fold higher than in DPSCs after 15 days in culture, and, almost 6-fold higher after 27 days. In addition, the expression of DSP, DMP-1 and ALP was observed in both DFCs and DPSCs, with a slow increase until 15 days of differentiation, and, after 21 days, there was a rapid increase in the expression of these genes in DFCs. In contrast, expression of these genes was almost undetectable in OMCs and in undifferentiated DFCs, DPSCs and OMCs. These results suggest that, compared to DPSCs, DFCs have a superior capacity for dentin formation and mineralization and that they may be useful for the regeneration of dentin or tooth tissues using tissue engineering technology.

Keywords :

dental follicle cells, dental pulp stem cells, odontoblast-related gene, real time RT-PCR, tissue engineering

Introduction

The emerging fields of tissue engineering and regenerative medicine focus on the replacement or repair of lost or damaged tissues (1). This tissue engineering and regeneration requires three key ingredients: morphogenetic signals, including growth and differentiation factors; responding stem cells; and a scaffold of extracellular matrix (1). We have previously studied the use of mesenchymal stem cells along with platelet-rich plasma for the regeneration of bone and mucosal tissue for dermis regenera-

tion (2-8). We try to extend these studies into the area of dental tissue regeneration.

After physiologic stimulation or injury, such as caries and operative procedures, pulp stem cells may be mobilized to proliferate and differentiate into odontoblasts by morphogens secreted from surrounding dentin matrix. The damaged odontoblasts can be replaced by newly generated populations of odontoblasts derived from residual cycling stem cells in the pulp (9). Also, the dental pulp tissue contains some stem cells that retain the potential to replicate and

differentiate into dentin-forming odontoblasts (10). In addition, during minor insults, odontoblasts differentiate into secondary dentin.

Dental follicle cells (DFCs) are one type of stem cells present in oral tissues that could be used for the regenerating of dental tissues. The dental follicle is a loose connective tissue sac that surrounds the unerupted tooth. The DFCs originate from ectomesenchymal cells, and they contain subpopulations of mesenchymal progenitors that give rise to periodontal tissue, including cementum, periodontal ligament, and alveolar bone. However, few studies have characterized the roles of progenitor cells derived from DFCs. Wise *et al.* reported that DFCs express many genes related to tooth eruption (11) and DFCs have been reported to have the ability to differentiate into cementoblasts (12), but whether they have the capacity to differentiate into dentin is unknown.

In addition to DFCs, oral tissues also contain a population of putative postnatal stem cells in the dental pulp termed dental pulp stem cells (DPSCs) (10). The DPSCs are clonogenic and highly proliferative cells that have an ability to regenerate a dentin-pulp like-complex found in normal human teeth, including a mineralized matrix containing odontoblast-lined tubules and fibrous tissue containing blood vessels. DPSCs also express several bone markers in a manner similar to bone marrow stem cells, which are known for their high proliferative capacity and ability to differentiate into multiple cell types (10) as well as their stem cell-like qualities, including the capacity for self-renewal and multi-linkage differentiation.

In the current studies, we examined whether DFCs and DPSCs can differentiate into odontoblasts by following the expression of specific genetic markers. Specifically, we investigated the expression of odontoblast-specific genes, including dentin sialophosphoprotein (DSPP) and dentin sialoprotein (DSP), as well as genes expressed in mineralized tissue, including dentin matrix protein 1 (DMP-1) and alkaline phosphatase (ALP) (13).

The DSPP gene is found only in dentin and is a marker of odontoblast differentiation. DSP gene is

transported *via* odontoblastic processes to the mineralization front shortly after synthesis. This gene is also a developmentally regulated protein that appears in terminally differentiated odontoblasts prior to the onset of mineralization (14).

ALP and DMP-1 are found not only in dentin but also in mineralized tissue. ALP is expressed constitutively by bone-forming cells and some periodontal ligament cells, and it is used as enzymatic marker of bone differentiation (15). DMP-1 was first cloned from the mineralized dentin matrix (16). Although the precise function of DMP-1 has not been determined, *in situ* hybridization experiments have shown that it is expressed in both osteoblasts and odontoblasts (17).

To study the expression of differentiation of DFCs and DPSCs, we used real-time reverse transcriptase-polymerase chain reaction (RT-PCR) (18) to analyze the expression of these four marker genes. RT-PCR allows accurate and reproducible determination of the gene copy number, and, unlike standard PCR, this method does not require post-PCR sample handling, preventing product carry-over contamination and increasing assay throughput (19). Our results show that DFCs and DPSCs might have the ability to form dentin and, therefore, may be useful for the regeneration of dentin or tooth tissues using tissue engineering technologies.

Materials and Methods

Cell culture and RNA collection

Normal human impacted third molars were collected from adults (13 to 15 years of age) at Nagoya University Hospital. All materials were used with the patients' informed consent. Dental follicle and pulp were separated by visual inspection immediately after extraction (Fig. 1). The isolated tissues were placed in 75 cm² flasks and cultured for 20 to 30 days in conditioning medium (CM; Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 0.05 units/ml penicillin) at 37°C in a humidified atmosphere containing 5% CO₂.

Cells obtained from the primary culture were

Fig. 1. Schema of experimental design.

plated in a 100 cm² tissue culture plate at a density of 3.1×10^3 cells/cm² (2.43×10^5 cells per dish). To induce these cells to form hard tissue, the medium was changed to induction medium (IM ; CM supplemented with 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 0.05 M ascorbic acid-2-phosphate). After 3, 6, 9, 15, 21, and 27 days, cultures were rinsed with phosphate-buffered saline (PBS) and total RNA was extracted using an RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany).

Analysis by real-time RT-PCR

Real-time RT-PCR was performed using a 7000 sequence detector (Perkin-Elmer, Wellesley, MA, USA). The reaction mixture was prepared according to the manufacture's protocol to give a final concentration of 1x avian myeloblastosis virus. Each 50 μl multiplex PCR reaction contained 200 ng RNA, Tfl reaction buffer, 0.2 mM dNTPs, 1.5 mM MgSO₄, 0.1 unit/ml avian myeloblastosis virus reverse transcriptase, and 0.1 unit/ml Tfl DNA polymerase probe (PCR-Access, Promega, Tokyo, JPN) as described by Gibson *et al.* (19). DNA amplification was performed with the following thermal cycling profile : 50°C for 2 min, 60°C for 30 min, 95°C for 5 min, then 50 cycles of 95°C for 20 sec, and final extraction at 60°C

Table 1. Sequences of Oligonucleotide primers and probes

primer of probe	sequence	position
DSPP 163F	GCCATTCCAGTTCCTCAAGC	163-187
DSPP 307R	CATGCACCAGGACACCACTT	307-288
DSPP probe	TGATGGTTCCACTGGCATTCTAACTCATCC	225-283
DSP 3083F	GTGAAATATCTGGCAAACGAGACA	3083-3106
DSP 3204R	CAGAGTTTCCAGTCCTGAGGTGTA	3204-3181
DSP probe	ATTGCTGAACTTTGCGCCAATTCA	3104-3127
hALP 783F	CCTCGTTGACACCTGGAAGAG	783-803
hALP 822R	TTCCGTGCGGTTCCAGA	822-806
hALP probe	TTCAAACCGAGATACAAGCACTCCCACTTC	784-813
DMP1 6F	GATCAGCATCCTGCTCATGTTC	6-27
DMP1 115R	GAGCCAAATGACCTTCCATT	115-95
DMP1 probe	CCTGTGCTCTCCAGTAACCAGGTATCAAA	41-71
GAPDH 1457F	GAAGGTGAAGGTCGGAGTC	1457-1475
GAPDH 3412R	GAAGATGGTGATGGGATTTC	3412-3392
GAPDH probe	CAAGCTTCCCCTTCTCAGCC	3363-3382

for 1 min. The primers and probes for real-time RT-PCR for DSPP, DSP, ALP, and DMP-1 were designed using Oligo version 4.0 (National Bioscience, Plymouth, MN, USA) according to Heid *et al.*(18). A reporter oligonucleotide probe labeled at the 5'-end with a fluorescent reporter dye and at the 3'-end with a fluorescence quencher dye was designed to bind to the product amplified by the oligonucleotide primer pair. The sequences of all oligonucleotides used are shown in Table 1. The GAPDH primer and probe (TaqMan GAPDH detection reagents) were purchased from Applied Biosystems (Applied biosystems, Foster, USA). Signals were analyzed with the sequence detector 1.0 program (Perkin-Elmer). The amount of each mRNA was determined by comparing the real-time RT-PCR signals to a standard curve of signal vs. human bone marrow t-RNA (Clontech, Palo Alto, CA, USA) at different concentrations (4000 ng, 2000 ng, 1000 ng, 500 ng, 250 ng, 125 ng, and 62.5 ng). The expression coefficients for each sample were determined by dividing the amount of specific mRNA by the amount of GAPDH mRNA.

Results

Morphology of DFCs, DPSCs, and OMCs in culture

We first observed the morphology of DFCs, DPSCs, and OMCs by phase contrast microscopy (Figs. 2A-C). These cells exhibited like spindle-shaped and fibroblastic form in IM. More bone nodules were found in DFCs than in DPSCs, and none were found in OMCs in IM.

Odontoblast-specific and osteogenesis-related gene expression in DFCs, DPSCs, and OMCs in culture

To investigate the ability of DFCs, DPSCs, and OMCs to form dentin, we examined the expression of odontoblast-specific (DSPP and DSP) and osteogenesis-related (ALP, DMP-1) genes using real time RT-PCR. Steady-state expression of GAPDH mRNA was used as a control for expression, and similar levels of expression were found throughout the different experimental conditions.

Expression of the DSPP gene was observed in

Fig. 2. Phase-contrast images of DFCs, DPSCs, and OMCs after 15 days in IM. DFCs: bone nodules are often found (A). DPSCs: appear as fibroblast-like cells (B). OMCs: bone nodules are not found (C). In all panels, magnification: 20X.

DFCs at later times (day 6) of growth in IM. The coefficient of expression in DFCs and DPSCs increased with time, but the expression of DSPP in OMCs was nearly undetectable. After 3, 6, 9, 15, 21, and 27 days in IM, the expression coefficients in DFCs were 0.28, 0.75, 1.08, 1.52, 2.85, and 5.80, respectively. Thus, the value at day 27 was 20.7-fold higher

DSPP

Fig. 3. DSPP gene expression in DFCs (open squares), DPSCs (striped squares), and OMCs (closed squares). After 3, 6, 9, 15, 21, and 27 days, cells were lysed, and total RNA was extracted. Equal amounts of total RNA (200 ng) were analyzed by real-time RT-PCR, and the amounts of mRNAs for DSPP and GAPDH in each sample were determined by comparing the signals to standard curves. The expression coefficient for mRNA on the ordinate was calculated for each sample by dividing the amount of DSPP mRNA by the amount of GAPDH mRNA. Each point is the mean value obtained from three independent experiments. And the differences were less than 10% in the three experiments. Each bar represents the mean, and the error bars represent the mean $\pm$ SD.

than the value at day 3. In DPSCs, we found expression of this gene after 15 days in IM, but the increase in expression was much slower. After 15 days, the expression coefficient in DFCs was also 6.17-fold higher than in DPSCs, and after 27 days, the expression coefficient was 6.26-fold higher in DFCs than in DPSCs. Finally, there was almost no expression of this gene in DFCs, DPSCs, or OMCs grown in CM (Fig. 3).

Expression of the DSP gene was observed in both DFCs and DPSCs grown in IM, with a slow increase until day 15. In DFCs, after 21 days in IM, there was a rapid increase in DSP gene expression. In DFCs, after 3, 6, 9, 15, 21, and 27 days in IM, the expression coefficients were 0.33, 0.39, 0.60, 0.86, 3.55, and 4.79, respectively. Therefore, the value after 27 days was 14.5-fold higher than the value after 3 days. In addition, the expression coefficient for DSP in DFCs was 4.55-fold higher than the value in DPSCs after 21, and 4.00-fold higher than after 27 days. Almost no

DSP

Fig. 4. DSP gene expression in DFCs (open squares), DPSCs (striped squares), and OMCs (closed squares). Experiments were performed and analyzed as in Fig. 3 except that DSP and GAPDH gene expression were measured.

expression of the DSP gene was found in OMCs in IM or in any of the three cell types in CM (Fig. 4).

Until 15 days of growth in IM, there was almost no expression of the ALP in the DPSCs and DFCs, but after 21 days, there was a time-dependent increase in the expression coefficient in DPSCs and DFCs. The expression coefficients were 0.49 and 1.15 and after 21 days and 0.98 and 4.37 after 27 days in DPSCs and DFCs, respectively. Therefore, after 21 days, the expression coefficient for ALP in DFCs was 2.35-fold higher than in DPSCs, and, after 27 days, 4.46-fold higher in DFCs than in DPSCs. Almost no ALP gene expression was observed in OMCs in IM or in any of the cell types in CM (Fig. 5).

Expression of the DMP-1 gene in DFCs and DPCs grown in IM appeared after 21 days and increased with time thereafter. The expression coefficients for DMP-1 gene expression were 0.70 and 1.29 after 21 days and 0.90 and 3.49 at 27 days in DPSCs and DFCs, respectively. Thus, the expression coefficient in DFCs after 21 days in IM was 1.84-fold higher than in DPSCs, and after 27 days, the expression coefficient was 3.88-fold higher than in DPSCs. There was almost no DMP-1 gene expression in OMCs grown in IM or in any of the three cells when grown in CM (Fig. 6).

ALP

Fig. 5. ALP gene expression in DFCs (open squares), DPSCs (striped squares), and OMCs (closed squares). Experiments were performed and analyzed as in Fig. 3 except that ALP and GAPDH gene expression were measured.

DMP-1

Fig. 6. DMP-1 gene expression in DFCs (open squares), DPSCs (striped squares) and OMCs (closed squares). Experiments were performed and analyzed as in Fig. 3 except that DMP-1 and GAPDH gene expression were measured.

Overall, these results show that DSPP, DSP, ALP, and DMP-1 genes were expressed in DFCs and DPSCs at later times when they were grown in IM. Expression of these genes was nearly undetectable in OMCs grown in IM, and in all three cell types grown in CM.

Discussion

In this study, we have examined the ability of DFCs, DPSCs, and OMCs to form dentin by following their expression of four dentin and bone extracellular matrix genes, DSPP, DSP, ALP and DMP-1. Our results show that these genes are expressed at later times when DFCs and DPSCs are cultured in IM, but are not expressed when DFCs and DPSCs are cultured in CM, and with different patterns. In phase contrast microscopy, bone nodules were found in DFCs and DPSCs, and none were found in OMCs in IM cultivation. This result might indicate that DFCs and DPSCs have the ability of forming the mineralized tissues.

High levels of DSPP expression have been shown to persist in functional odontoblasts throughout all phases of primary dentinogenesis and in secondary dentinogenesis (17). Also, the expression of DSPP mRNA has been shown to be tooth-specific, to remain high in odontoblasts during and after completion of primary dentinogenesis, and to be more fundamentally involved than DMP-1 in the formation and maintenance of dentin. DSPP transcripts have also been detected in polarizing odontoblasts and in functional odontoblasts depositing the extracellular matrix components of predentin-dentin (20). Furthermore, DSPP and DMP-1 participate in a regulatory pathway required for the growth and differentiation of odontoblasts (21) and play a role in the mineralization of dentin (22).

In the current study, we showed that DSPP gene expression in DFCs and DPSCs occurs at early time points in IM (6 days), whereas DMP-1 and ALP gene expression appeared after 15 days. We also showed that expression of these genes in DFCs and DPSCs increased with time. Therefore, based on DSPP and DMP-1 expression, DFCs and DPSCs may correspond with the mineralization of odontoblasts. We further showed that the expression coefficient of DSPP in DFCs was 6.17-fold higher than in DPSCs after 15 days in IM, and 6.26-fold higher after 27 days. This suggests that, compared to DPSCs, DFCs have a superior ability to form dentin and contain more pre-odontoblast cells.

Recently, *in situ* hybridization studies have demonstrated that DMP-1 is expressed in odontoblasts, ameloblasts, cementoblasts, and osteoblasts. It was also noted that DMP-1 is expressed not only in dentin but also on other mineralizing tissues (23). In the current study, we found that DMP-1 expression in DFCs and DPSCs increases at later times in IM. Therefore, DFCs and DPSCs may have the ability to mineralize at these later times. This is also supported by the expression of ALP in DPSCs and DFCs after 21 days in IM because this gene is an early marker for both osteoblasts and odontoblasts, plays a vital role in calcified tissue formation, and is thought to induce odontoblast differentiation (24). These results suggest that DFC and DPSC cultures contain cells that can differentiate into osteoblasts and odontoblasts after long times of continuous culture in IM. Finally, the patterns of DMP-1 and ALP gene expression were very similar, suggesting that their expressions are somehow related.

There was nearly no expression of the DSPP, DSP, ALP, or DMP-1 genes in OMCs or in DFCs or DPSCs grown in IM. This suggests that the cultivated cells might be differentiated into osteoblastic or odontoblast-like cells by the IM. This implies that the three supplements (dexamethasone, β -glycerophosphate, and ascorbic acid-2-phosphate) must be added to the CM to give the DFCs or DPSCs the ability to form dentin or bone. And there may be no stem cells in OMCs which was stimulated to form mineralized tissue by IM.

In mice, the expression of DSP in dental pulp appears after odontoblast terminal differentiation and concurs with pre-dentin formation before the onset of mineralization (14). *In situ* hybridization in adjacent tissue sections show that DSP is deposited when dentin components are secreted and are progressively converted into dentin. However, the function of DSP remains unclear. In the current study, DSP gene expression in DFCs was observed after 21 days of growth in IM, and the gene expression pattern was similar to that of ALP and DMP-1 but different than DSPP. Based on these findings, we speculate that the DSP gene is induced at the initial

phase of and participates in dentin mineralization.

In conclusion, our data demonstrate that DFCs should have the ability to differentiate into odontoblasts and that they contain more pre-odontoblast cells than DPSCs. Based on their expression of the DSPP, DSP, and DMP-1 genes, DFCs and DPSCs may have the ability to form dentin. In the future, DFCs may be useful in the engineering of dental materials and in the preservation and regeneration of dentin and teeth.

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