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Expression of DSPP mRNA During Differentiation of Human Dental Pulp-derived Cells (HDPC) and Transplantation of HDPC Using Alginate Scaffold

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Tissue stem cells are used for the regenerative medicine. In previous study we observed hard tissue formation of human dental pulp-derived cells using alginate scaffold. In this study, we explore the ability to differentiate of the 13th passage cells with glycerol 2-phosphate disodium salt hydrate (β-GP) which accelerate calcification. Reverse transcriptase Polymerase Chain Reaction (RT-PCR), transplants using alginate scaffold and histological examination were performed. We observed the expression of DSPP mRNA on day 10 cultured cells with β-GP. In conclusion, the 13th passage cells still have an ability to differentiate into odontoblast-like cells and alginate supports the differentiation of cultured cells in the transplants.

Keywords: Odontoblast-like cell, Human, Dentin sialophosphoprotein Cell culture, Transplantation

Introduction

There have several studies on tissue stem cells for the regenerative medicine. It was elucidated that cultured mesenchymal cells differentiated the bone, cartilage, muscle, tendon, fatty tissue and the bone marrow stroma (Tuan *et al*., 2003; Pittenger *et al*., 1999; Award *et al*., 2000; Wakitani *et al*., 1995; Majumdar *et al*., 2000). On the other hand, some studies have demonstrated the appearance of odontoblast-like cells in the human dental pulp cell culture (Kim *et al*., 2005). Previously we subcultured human dental pulp-derived cells for several passages, transplanted the cells with alginate scaffold under the skin of nude mice, and observed the hard tissue formation in the transplants (Kim *et al*., 2005; Gronthos *et al*., 2000).

Dentin phosphoprotein (DPP), a highly acidic protein and the major non-collagenous component of dentin, is expressed by the ectomesenchymal derived odontoblast cells of the tooth. It is suggested that deficiency of the protein is a causative factor in *dentinogenesis imperfecta*. Dentin sialoprotein (DSP) is a 95-kDa Glycoprotein within the dentin extracellular matrix (DECM), and DPP is the major noncollagenous DECM protein (MacDougall *et al*., 1997). It has been elucidated that there are two major non-collagenous dentin matrix proteins, DSP and DPP (also known as phosphophoryn) encoded by a single gene termed dentin sialophosphoprotein (DSPP) Papagerakis *et al*., 2002; Narayanan *et al*., 2004). DSPP is in particular mapped to human chromosome 4 using a somatic cell hybrid panel (MacDougall *et al*., 1997). Whereas most of the previous regenerative medicine studies were designated on primary and young subcultured cells, in the present study we examined the expression of DSPP mRNA, the relation between the expression and the differentiation to identify the property of the sub-cultured human dental pulp-derived cells. Using cultured cells derived from human dental pulp would be helpful in further elucidating the mechanisms of

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odontoblast differentiation and the mineralization process (Couble *et al.*, 2000).

Material and Method

Preparation of dental pulp cells

The human dental pulp was sterilely dissected from teeth removed for treatment purpose at the Hospital of Osaka Dental University. The protocol was approved by the Ethics Review Board at Osaka Dental University (no. 050355: culture of human dental pulp stem cells). The cells were isolated by collagenase type I (4 mg/ml; Wako Pure Chemical Industries, Osaka Japan) and dispase (4 mg/ml; Gibco Laboratories, Grand Island, NY, USA) at 37°C for 40 minutes. The solution with isolated cells was filtrated with the Cell Strainer (70 μm; BD Falcon, Bedford, MA, USA), centrifuged (1,500 rpm, 3 min) and added with Dulbecco's Modified Eagle Medium (DMEM, Nacalai Tesque, Inc., Kyoto, Japan) containing penicillin (100 units/ml; Gibco), streptomycine (100 μg/ml; Gibco) and Fetal Bovine Serum (20%; FBS, HyClone, Logan, UT, USA). The primary culture was seeded on dishes (35 mm; BD Falcon) and cultured under 5% CO_2 gas at 37°C. While confluent occurred, the cells were seeded consequently at 5,000/cm^2 cell concentration by gradient reduction of 10%FBS every 3rd day in every succeeding passage. After 2 months of cell subculture, the 13th passage cells were obtained.

Examination of cell differentiation

Glycerol 2-phosphate disodium salt hydrate (β-GP; SIGMA-ALDRICH GmbH, Steinheim, Germany) was added to the experimental group cultures. Each culture was seeded with 10,000 cells/cm^2 using 24-well plates (Asahi Technoglass Co., Chiba, Japan).

1. Alkaline phosphatase activity

The alkaline phosphatase (ALP) enzyme activity of 3, 7, 14, and 21 days old subcultured cells was examined using an Alkaline Phosphatase Substrate Kit (Bio-Rad Laboratories, Hercules, CA, USA) following the directions. Afterward, the specimens were fluorescent -stained with Bisbenzimide H 33258 (Wako), and the fluorescent intensity (excitation wave length: 355 nm, detection wave length: 460 nm) was summarized and scored using the multilabel counter (WALLAC OY). The ALP activity was corrected by the DNA amount.

2. Formation of calcifying loci

For examination of calcification loci on the 3, 7, 14, and 21 days subcultures, they were fixed with formalin (10%) and then stained with alizalin red S (1%; SIGMA-ALDRICH JAPAN).

Transplantation

13th passage dental pulp-derived cells (1.5×10^7 cells/well) were made to be a cluster by mixture with alginate solution (1.5%, 0.3 ml; Protanal FL 10/60 Sodium Alginate; FMC Bio Polymer, Drammen, Norway) and $CaSO_4$ (21%, 40 μl). The cell clusters were transplanted subcutaneously and epifascically into the dorsolumbar portion of KSN/Slc-nu/nu nude mice (7week-old, male; Japan SLC, Inc., Hamamatsu, Japan). Implantation of alginate alone was performed in the control group. After 6 weeks of the transplantation, Softex (soft X-ray) photography was con-ducted for detection of hard tissue formation (25 kVp, 20 mA, 10 sec; Industrial X-Ray Film FR, Fuji Film Co., Ltd.). Subsequently, the mice were fixed by intra-cardiac perfusion with paraformaldehyde (4%), the back tissue with implants were dissected and processed for light and transmission electron microscopy purpose, respectively.

Histological examination

H-E staining was routinely proceeded. For the immunological staining, mouse monoclonal collagen type I antibody (COL-1) (Abcom Limited, Cambridgeshire, UK) and Rabbit Anti (bovine) type I Collagen (LSL Co., LTD., Tokyo, Japan) were used. TUNEL method was conducted on paraffin sections using ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (CHEMICON Intl, Inc., Temecula, CA, USA) for the investigation of cell turnover in the transplants and was observed with confocal laser scanning microscopy (CLSM). Observation with transmission electron microscopy (TEM) was routinely proceeded.

Property of 13th passage cells

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The examination was performed on the 10th day after adding β-GP. First-strand cDNA synthesis was performed by using SuperScript™ III CellsDirect cDNA Synthesis System (Invitrogen life technologies, Cattahad, CA, USA). Specific primers were designed according to the previous study (Table 1, Fujimoto *et al.*, 2001). First-strand cDNA (2 μl) was diluted in a PCR reaction mixture (10×PCR ExTaq Buffer, 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, 0.25 units of TaKaRa ExTaq™; TaKaRa BIO, 10 pmol of

Table 1

	Size	Primer	
dentin sialophosphoprotein (DSPP)	248bp	forward	5'-GAT GAT CCC AAT AGC CA-3'
		reverse	5'-CCT TTG CCA CTG TCT G-3'
glyceraldehydes-3-phosphate dehydrogenase (G3PDH)	450bp	forward	5'-ACC ACA GTC CAT GCC ATC AC-3'
		reverse	5'-TCC ACC ACC CTG TTG CTG TA-3'

each human specific primer sets; SIGMA-ALDRICH JAPAN). Amplification was performed in a thermal cycler (TaKaRa PCR Thermal Cycler PERSONAL). This amplification system for DSPP included 94°C (2 min), 40 cycles of 94°C (45 sec), 55°C(45 sec) and 72°C (1 min), followed by 10 min at 72°C. Ampli-fied sample (5 μl) was analysed by 1.5% agarose gel electrophoresis at 100 V for 30 min. After electrophoresis, the gel was stained with ethidium bromide solution (0.2 μg/ml) for 30 min. Stained gels were observed under ultraviolet light (ATTO Printgraph; ATTO, Tokyo, Japan).

Results

Examination of the monolayer cultures

1. ALP activity

No significant ALP activity and differences were evident in the 3 and 7 days subcultured cells between control (β-GP(-)) and experimental (β-GP(+)) groups. However, ALP activity was observed in both groups on the 14th day. Furthermore, we observed that the ALP activity of β-GP(+) group became distinct as twice as higher than the β-GP(-) group on the 21st day of subculture (Fig. 1).

2. Observation of Calcification loci

Occurrence and concrescence of calcification loci had been observed since the 14th day of subculture composed of isolated proliferating and differentiating dental pulp cells (Fig. 2).

Examination of the transplant in situ

1. Softex radiography revealed radio-opeque calcified bodies in the subcutaneously transplanted cell/alginate clusters. We dissected the transplant with surrounding tissue and observed some whitish hard granules in the transplant.

2. Histology of the tranplants

1) Light microscopy

(1) Control group: transplantation of alginate scaffold into the backs of nude mice

Histological findings of the H-E stained specimens showed a network of fibroblast strands extending between the cutis and transplant. By using mouse monoclonal collagen type I

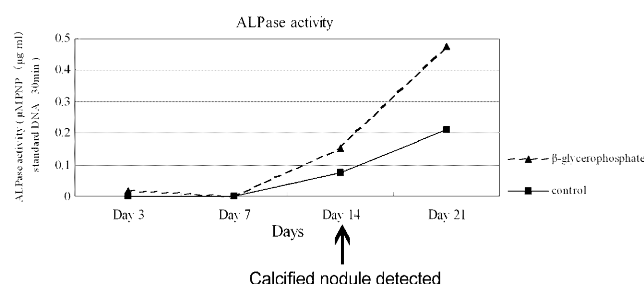

Fig. 1. ALP activity of the cultured cells. On the day 21 of subculture, ALP activity in β-GP(+) group is twice as high as in β-(-) group.

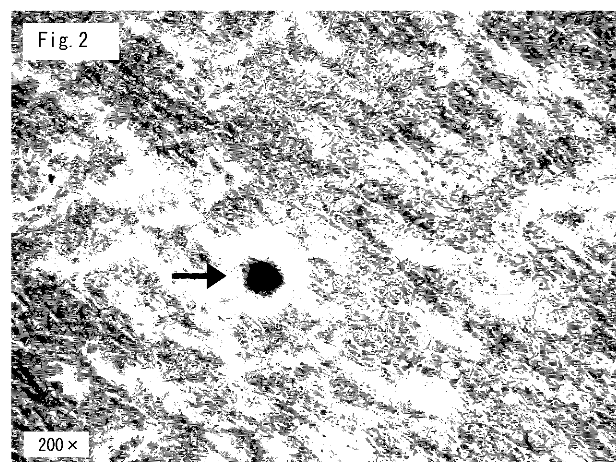

Fig. 2. Calcification loci of the cultured cell (Alizarin Red staining). Occurrence and concrescence of calcification loci (arrow) was observed since day 14 of subculture.

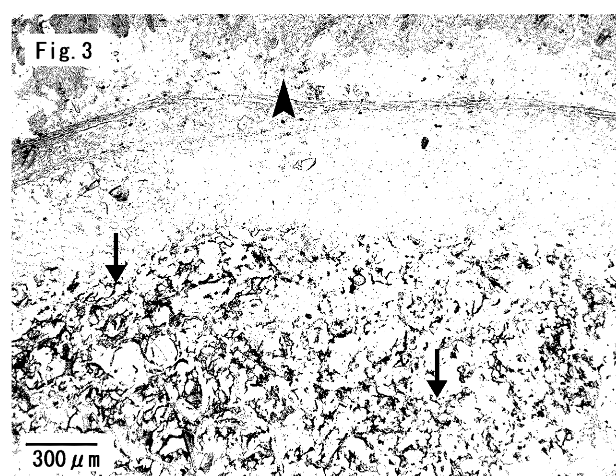

Fig. 3. Immunohistochemistry of type I collagen (arrowhead points epidermis) in the control group. Type I Tcollagen (+) immunoreactions are observed in the implants.

antibody, distribution of Type I collagen immuno-reactivity was evident in the cell-strand network in the transplants (Fig. 3).

(2) Experimental group: transplantation of the cell cluster (dental pulp-derived cells with the alginate scaffold) into the backs of nude mice

Routine H-E staining specimens showed extracellular matrix (ECM) formation in transplants containing sub-cultured 13th passage cells and alginate scaffold (Fig. 4). Immuno-reactivities of mouse monoclonal collagen type I antibody and Rabbit Anti (bovine) Type III Collagen was evident in the transplants (Fig. 5). Monoclonal Antibody to Bovine Osteocalcin localized osteocalcin in the ECM. No vascular system extension between the cutis and the ECM was demonstrated by Polyclonal Rabbit Anti-human Von Willebrand factor (factor VIII), which stains endothelial

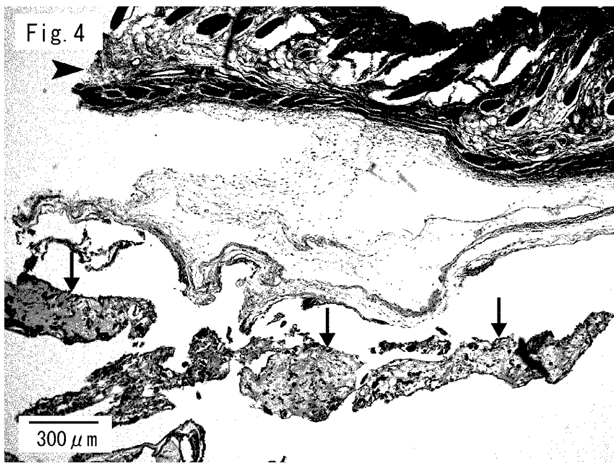

Fig. 4. Photomicrograph of a transplant and surrounding tissue (H-E staining; an arrow head: cutis). Extracellular matrix (ECM) formation is identified in the subcutaneous transplant containing 13th passage cells/alginate scaffold *in situ* (arrows).

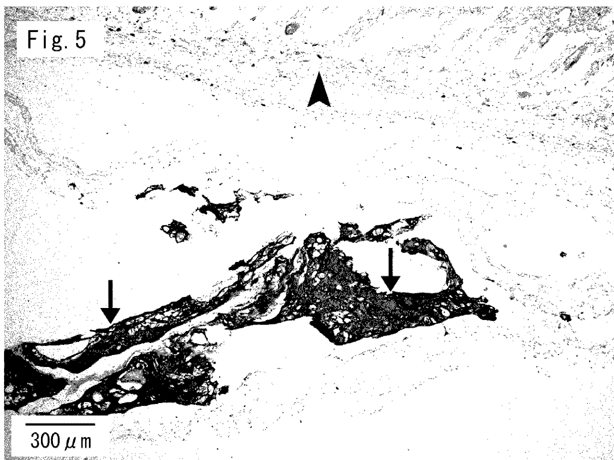

Fig. 5. Immunohistochemistry of type I Tcollagen (arrow head: epidermis). Type I collagen is observed in the transplant (arrows).

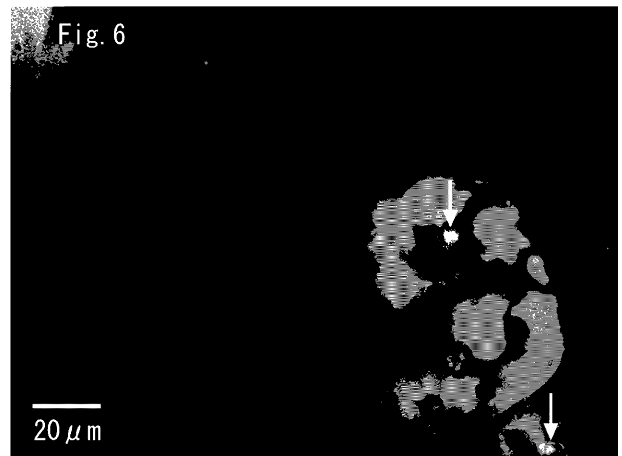

Fig. 6. DSP (+) reaction was observed in the transplant by confocal laser scanning microscopy. DSP (+) reaction suggests the dentin-like tissue formation.

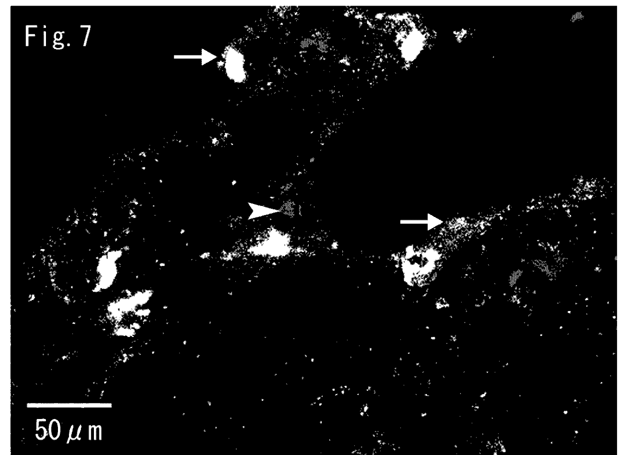

Fig. 7. The transplant in situ TUNEL (+) cells were observed in the calcifiying transplant by confocal laser scanning microscopy.

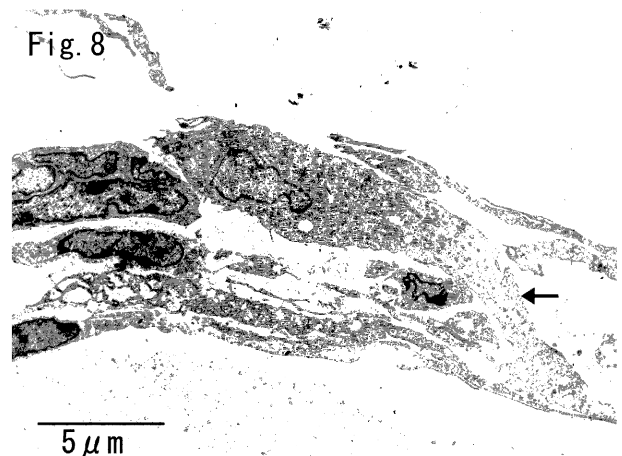

Fig. 8. Transmission electron microscopic appearance of a transplant. Isolated and polarized cells with a distal cell process (arrow) contain abundant cytoplasm; the intercellular junctional apparatuses are not well developed.

cells.

2) CLSM

Cell turnover by the occurrence of TUNEL (+) cells in the experimental group was evident in the calcifying transplant containing dental pulp-derived cells and the alginate scaffold (Fig. 6). Non-collagenous protein DSP(+) reaction was observed in the transplants (Fig. 7).

3) TEM of the transplant

Fine structure study of the ultrathin-sectioned demineralized specimens revealed isolated spindle-shaped, elongated and polarized fibroblast-like cell in the trans-plants; the intercellular junctional apparatuses of the cells were not well developed. The polarized preodontoblast-like cells were abundant in cytoplasm having a distal extending main cellular process. Scattered autolyzing cells were found in the transplants (Fig. 8).

In addition, at the periphery of the calcified matrix, certain apocrine secretion of the matrix vesicles-like structures was also observed.

Examination of the property of the transplanted cells

1. RT-PCR

No expression of DSPP mRNA was found in the β-GP (-) primary passage cells or succeeding 12 passages of cells on the 10th day culture. However, in the β-GP (+) group expression of DSPP mRNA was detected in the 13th passage cells on the 10th day culture.

In the present RT-PCR study, positive control amplifications were performed using a primer set for the housekeeping gene, glyceraldehydes 3-phosphate dehydrogenase (G3PDH). Negative controls were performed using each specific primer set with the added cDNA target replaced by sterile water. Expression of DSPP mRNA was not found in the β-GP(-) primary and succeeding 13 passages. On the contrary, by adding β-GP into the media, DSPP mRNA expression was observed on the 10th day after subculture (Fig. 9). In the control group, DSPP mRNA expression was observed on the 23rd day after subculture (Fig. 11). But in the β-GP adding group, DSPP mRNA expression was not observed on the 23rd day after subculture (Fig. 10).

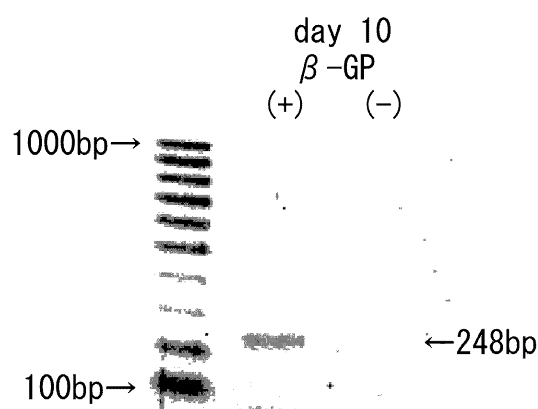

Fig. 9. RT-PCR analysis of expression of DSPP mRNA on day 10. DSPP mRNA was expressed in the β-GP (+) group, but not in the β-GP (-) group.

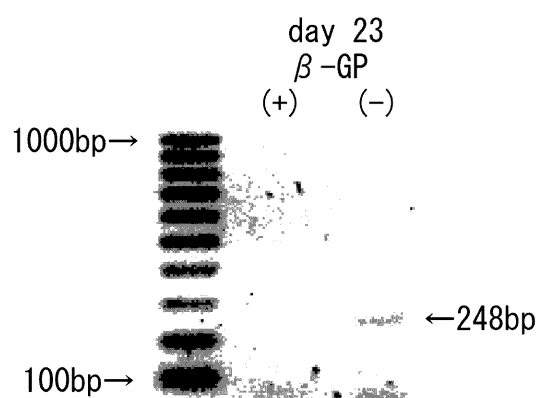

Fig. 10. RT-PCR analysis of expression of DSPP mRNA on day 23. DSPP mRNA was expressed in the β-GP (-) group, but not in the β-GP (+) group.

Discussion

There are several studies that showed the differentiation of odontoblast-like cells with matricial calcification by culture of dental pulp-derived cells from the rat, human and cattle teeth (Couble *et al.*, 2000; Kasugai *et al.*, 1993; Seux *et al.*, 1991; Nakashima, 1991). The odontoblast-like cells were characterized by cell polarization and distal extension of the main cell process. Additionally, the expression of DSPP that encode DSP and DPP related to the dentin specific protein was observed in these differentiated cells found in dentin (Kasugai *et al.*, 1993; James *et al.*, 2004; Begue-Kirn *et al.*, 1998). On the other hand, many studies have conducted on factors inducing mesenchymal stem cells to initiate calcifying tissue. The studies indicated that the bone formation was induced by dexamethasone, β-GP, vitamin D, ascorbic acid and BMP, while the cartilage formation was induced by dexamethasone, ascorbic acid, TGF-β (Couble *et al.*, 2000). A previous study has reported that β-GP was particularly essential in inducing the cultured dental pulp-originated cells to acquire the odontoblast property (Kasugai *et al.*, 1993).

In tooth formation, the odontoblasts, highly specialized cells aligned in a single layer at the edge of the dental pulp, are responsible for secretion and mineralization of the fibrillar extracellular matrix of dentin. They are originated from mesenchymal dental papilla cells different degrees of differentiation. They withdraw from the cell cycle, cellular polarization, formation of a cellular process, and synthesis and secretion of specific proteins. Among those, dentin DSP and dentin DPP are dentin specific and others like type I Tand type I Ttrimer collagen, osteocalcin, osteopontin, dentin matrix protein (DMP1) are common to both dentin and bone (Saito *et al.*, 2004).

DSPP gene encodes two dentin-specific proteins: DSP and DPP. The study in rodent incisor, a small amount of DSPP mRNA was detected in young odontoblasts secreting predentin. Immunolocalization of DSP and DPP in young and mature odontoblasts and in dentin suggested that these proteins were important in dentinogenesis and dentin mineralization (Saito *et al.*, 2004). DPP appears to be secreted at the mineralization front, binds to collagen fibers in the gap regions, and *in vitro* experiments showed that it initiated apatite crystal formation, but its function *in vivo* remains unclear (Couble *et al.*, 2000). We performed RT-PCR to investigate DSPP mRNA expression by human dental pulp cells as a differentiation marker of odontoblasts in the presence of β-GP. The expression of DSPP mRNA was detected on the 10th day. Also, DSP was detected within the transplants with a dentin-like hard tissue formation. The

findings indicate the dentin-like formation in the transplants containing cultured dental pulp-derived cells.

In the present study, we cultured 13th passage of human dental pulp cells and obtained certain fibroblast-like cells. ALP activity was not found except in succeeding β-GP (+) cultures. ALP activity and the expression of DSPP mRNA induced by β-GP indicated that odontoblast-like cells differentiated in the 13th passage cells. And the expression of DSPP mRNA on the 23rd day in the β-GP (-) group indicated that β-GP accelerate the differentiation of odontoblast-like cells. Also a previous study indicated that bone morphogenetic protein (BMP) and dexamethasone induce tissue formation and up-regulate the expression of DSPP mRNA (Ritchie *et al*., 2004; Tsukamoto *et al*., 1992).

Furthermore, we found that the ALP activity expressed in both groups between the 10th and 14th day, and in the experimental group, it became evident on the 21st day of culture. On the other hand, calcifying loci were demonstrated in the 14th day; it was similar to a previous study (Tsukamoto *et al*., 1992). Additionally, in the present results, we observed human cell cultures contained strands of intermingled cells at both proliferation, differentiation and apoptotic phases in the transplants.

The present study observed differentiation of odontoblast-like cells and dentin-like tissue formation in the transplants. Thus, alginate scaffold supports the differentiation of cultured cells in transplants. However, the transplants initiated dentin-like tissue formation in a specific environment different from the usual dentinogenesis. Further studies to clarify the mechanism of dentin-like tissue formation and succedent remolding of the transplants are next attempts we should confront.

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