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Molecules and Cells

The Presence of Open Dentinal Tubules Affects the Biological Properties of Dental Pulp Cells *Ex Vivo*

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To investigate the effects of open dentinal tubules on the morphological and functional characteristics of dental pulp cells. Morphological changes in human dental pulp cells that were seeded onto dentin discs with open dentinal tubules were investigated on days 1, 2, 4, and 10 of culture using scanning electron microscopy and fluorescence microscopy. Samples collected on days 1, 3, 6, 8, and 10 of culture were evaluated for cell proliferation rate and alkaline phosphatase activity. Cultured human dental pulp cells developed a columnar or polygonal morphology and monopolar cytoplasmic processes that extended into the dentinal tubules. The cells formed a multilayer and secreted an extracellular matrix onto the cell surface. Scanning electron microscopy and fluorescence microscopy revealed polarized organization of odontoblasts. Cells seeded onto dentin discs proliferated minimally but showed high levels of ALP activity. Dental pulp cells seeded onto treated dentin discs develop an odontoblast-like phenotype, which may be a potential alternative for use in experimental research on dentinogenesis.

INTRODUCTION

Odontoblasts play a central role in dentinogenesis (Linde and Lundgren, 1995). Various dental injuries cause localized odontoblast destruction. During the repair process, dental pulp stem cells (DPSCs) may be recruited and differentiate into odontoblasts or odontoblast-like cells to induce reactionary or reparative dentinogenesis. With advances in cytobiology, attention has been drawn to utilizing tissue-engineering techniques to regenerate dentin. A variety of bioactive molecules have been used to regenerate dentin or dentin-pulp complexes in combination with dental pulp cells, including transforming growth factor-β1 (TGF-β1) (Couble et al., 2000), insulin-like growth factor-1, bone morphogenetic protein (Lin et al., 2007), and dentin matrix protein (Hao et al., 2007).

Scaffolds, cells, and bioactive molecules are needed to regenerate dentin using tissue-engineering techniques (Nakashima and Akamine, 2005; Peng et al., 2009). Scaffolds derived from natural sources such as collagen, hydroxyapatite (HA), and hydroxyapatite/tricalcium phosphate (HA/TCP) are frequently used in three-dimensional forms to regenerate dentin. Dentin-pulp complexes have been produced in mice by transplanting DPSCs onto a collagen scaffold (Prescott et al., 2008).

Dentin discs have been used to mimic *in vivo* conditions because of their configuration and composition. Immortalized bovine pulp cells seeded onto a treated dentin surface had a proliferation rate similar to that of pulp cells that were seeded onto slides; in addition, they exhibited multipolar processes extending into the dentinal tubules but did not have an odontoblast-like morphology (Schmalz et al., 2001). Another study showed that DPSCs inoculated into dentin discs display odontoblastic morphological characteristics in which the unipolar processes of some cells extended into the dentinal tubule (Huang et al., 2006a). Remarkably, TGF-β1 stimulated odontoblasts to synthesize reactionary dentin and upregulated the expression of type I collagen in the dentinal tubules of thick slices of teeth. The studies mentioned above indicate that dentinal tubules may be key mediators of dentinogenesis (Magloire et al., 2001).

However, little information is available regarding the function of dentinal tubules during cell differentiation. In this study, we applied various treatments to dentin discs, observed the differentiation of dental pulp cells into odontoblast-like cells, and measured cell growth rate and alkaline phosphatase (ALP) activity.

MATERIALS AND METHODS

Cell culture

Cell cultivation was performed according to our previous reports (Cheng et al., 2010). After informed consent, impacted third molars were collected from healthy adults aged 20, 26, and 28 years. The pulp tissue or periodontium was gently separated, minced using scalpels, and then digested in 3 mg/ml of collagenase type I (Sigma-Aldrich, USA) for 1 h at 37°C. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 10% fetal calf serum (FCS; Gibco, USA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells that had undergone four to eight passages were selected, digested using pancreatin (2 g/L trypsin and 0.2 g/L EDTA), and seeded onto slides or dentin disc surfaces in a 24-well plate at a concentration of 1×10^4 cells per well.

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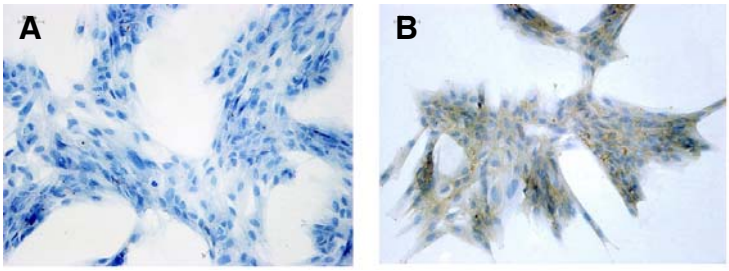

Fig. 1. Immunohistochemical investigation of the origin of dental pulp cells. (A) Immunolabeling for keratin was negative. (B) Vimentin-positive cells displayed a brown cytoplasm. (Original magnification: × 200; bar = 50 μm.)

Specimen preparation

Impacted third molars were collected from healthy adults (aged 17-23). Immediately after extraction, a diamond-coated band saw (Struers Minitom; Struers, Denmark) was used to separate the coronal dentin from the roof of the pulp chamber and to cut sections of approximately 10 mm × 7 mm × 0.5 mm. The dentin surfaces were then ground flat and hand-polished using aqueous slurries of progressively finer grades of silicon carbide, up to 4000 grit (Struers), thereby removing about 150 μm from the original dentin surface.

Prepared dentin discs were treated with 17% EDTA for 10 min and 19% citric acid for 1 min to remove the smear layer (Froes et al., 2000). The dentin discs were then soaked in 17% EDTA for 1 week at 37°C in a humidified chamber to induce demineralization of the dentin surface and to open the dentinal tubules. Dentin discs were immersed in 5.25% NaOCl for 24 h to sterilize them and to reduce the effects of inherent bioactive molecules. Specimens were rinsed and soaked with 1× phosphate-buffered saline (PBS) for 1 week to remove residual agents and dissolved dentin matrix components. They were then stored in serum-free medium.

Immunohistochemistry

Cells were washed with PBS, fixed in 4% paraformaldehyde for 15 min, treated with 0.1% Triton X-100 for 5 min, and blocked with 0.5% bovine serum albumin (BSA) for 1 h. The BSA was then removed and the cells were incubated with primary antibody diluted in PBS for 2 h at 37°C in a humidified chamber. They were then rinsed in PBS and incubated with biotin-labeled goat anti-mouse IgG (1:100) for 20 min at 37°C. After washing with PBS, the cells were incubated with phytomycin avidin conjugated with peroxidase (1:100) for 30 min at 37°C and then washed with PBS again. Diaminobenzidine was added for color development, after which the cells were stained for 2 min with hematoxylin, dehydrated, and mounted on slides using neutral gum. Images were acquired using a microscope. Anti-vimentin antibody (1:50), anti-keratin antibody (1:200), anti-CD45 (1:100), and anti-CD34 (1:200) were obtained from Santa Cruz Biotechnology Inc. (USA).

Immunofluorescence

Cells were collected on days 1, 2, 4, and 10. They were washed three times in PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, and then treated with 0.1% Triton X-100 for 5 min at 37°C. Fibrous actin (F-actin) was visualized using fluorescein isothiocyanate (FITC)-phalloidin (Sigma-Aldrich, Germany). Images of dental pulp cells seeded onto dentin discs and on glass slides were acquired using a fluorescence microscope (Carl Zeiss, Germany).

Scanning electron microscopy (SEM)

Samples were collected on days 1, 2, 4, and 10. For SEM analysis, cells were washed three times with PBS, fixed with 2.5% glutaraldehyde at 4°C for 2 h, dehydrated in a graded series of ethanol for 20 min, interchanged in isoamylacetate for 15 min, and air-dried. After gold coating, the preparations were observed using an SEM (Carl Zeiss). Periodontal fibroblasts that were incubated on dentin discs and slides as described above were used as controls.

MTT assay

Dental pulp cells were seeded onto dentin disc surfaces in a 24-well plate at a concentration of 1×10^4 cells per dentin disc in MTT solution (2 mg/ml growth medium), incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 4 h, and washed with PBS, after which 420 μl of dimethyl sulfoxide (DMSO) was added to each well. Absorption at 570 nm (OD570) was determined spectrophotometrically. The medium group and mineral liquid group were used as control groups (mineral liquid: β-phosphoglycerol, 2.16 g/L; dexamethasone, 3.295 μg/L; vitamin C, 50 mg/L; amphotericin B, 0.3 mg/L; $NaHCO_3$, 2.2 g/L; L-glutamine, 0.219 g/L; hepes, 2.38 g/L; DMEM medium, 1 L) and were dealt with in the same way.

Alkaline phosphatase activity assay

Dental pulp cells were seeded onto dentin disc surfaces in six-well plates at a density of 1×10^4 cells per dentin disc. Cells were harvested on days 1, 3, 6, 8, and 10. ALP activity was determined using an ALP kit (Baiding, China) according to the manufacturer's protocol. The medium and mineral liquid groups were used as controls.

Statistical analysis

Data analysis was performed using one-way analysis of variance (ANOVA). Results are expressed as the mean ± S.E.M. A *P*-value of < 0.05 was considered statistically significant. All experiments were repeated at least twice.

RESULTS

Identification of the source of dental pulp cells

Keratin is mainly expressed by epidermis cells. Vimentin is expressed by fibroblast-like mesenchymal cells, vascular smooth muscle cells, and mesoderm-derived cells. Figure 1 shows that cultured cells were negative for keratin and positive for vimentin. This indicates that the cultured cells were derived from the mesenchyme.

Identification of DPSC surface markers

Immunoreactivity profiles of the cultured cells were conducted to detect stem cells. CD34 is a surface marker of mesenchymal stem cells and hematopoietic stem cells. CD45 is a surface marker of hematopoietic stem cells (Pittenger et al., 1999; Zhang et al., 2003). Immunohistochemical staining showed that CD34 was expressed in passage 4 (Fig. 2A) and passage 8 (Fig. 2C), whereas CD45 was not expressed (Figs. 2B and 2D).

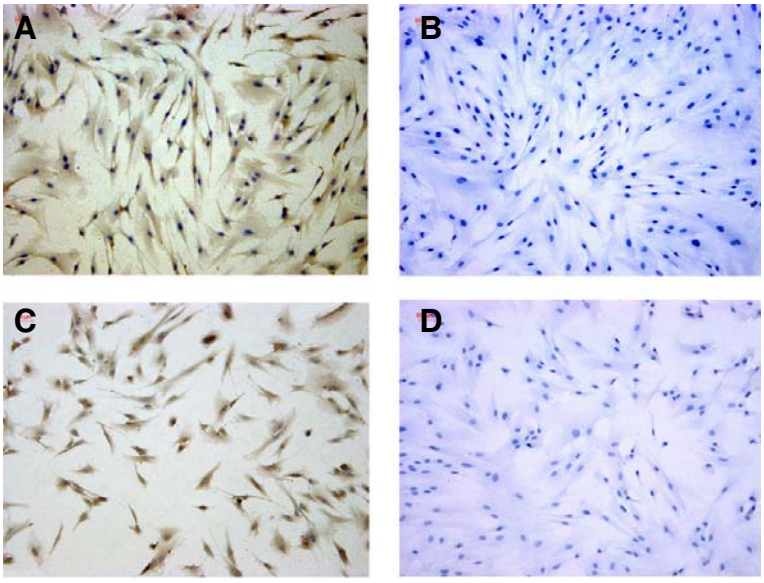

Fig. 2. Immunolabeling of DPSC surface markers. (A) Labeling for CD34 shows a high degree of fluorescence in the cytoplasm during passage 4. (B) Staining for CD45 was absent during passage 4. (C) CD34-positive cells displayed a brown cytoplasm during passage 8. (D) Staining for CD45 was absent in the cytoplasm during passage 8 (Original magnification: × 100; bar = 50 μm).

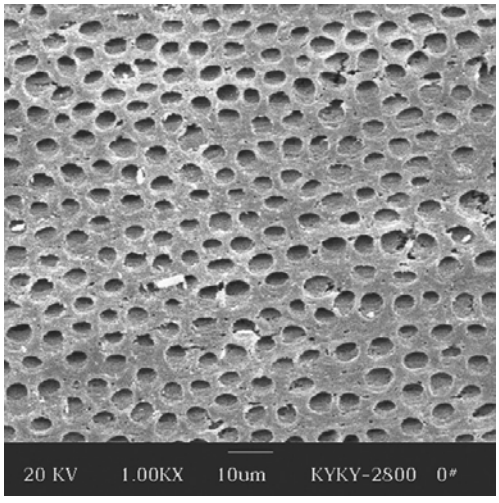

Fig. 3. Morphology of treated dentin discs. Scanning electron microscopy image showing the orifices of dentinal tubules (Original magnification: × 1000; bar = 10 μm).

This demonstrates that mesenchymal stem cells exist among cultured dental pulp cells and are maintained as a subculture.

Morphology of the orifices of dentinal tubules

SEM analysis of processed dentin discs (Fig. 3) showed that the orifices of dentinal tubules were intact and completely open; they had a shallow patellate appearance and a diameter of about 5 μm.

Growth of dental pulp cells on dentin discs

The morphological changes of cells on the dentin discs were visualized using SEM. On day 1, short fusiform cells attached to the dentin. On day 2, the processes of cells spread out and some cells formed a monopolar cytoplasmic process extending into an open dentinal tubule. On day 4, the upper cells on the dentin discs appeared elongated and had formed a multilayer. Figure 4C shows that the cell bodies were columnar or polygonal and that clusters of columnar cells were arranged in parallel lines, which were probably associated with polarized cellular characteristics. On day 10, the morphology of the cells was similar to that on day 4, but some punctate substance on the cell surface was observed. In contrast, dental pulp cells that were incubated on slides did not form a multilayer (data not shown).

Periodontal ligament cells were also seeded onto dentin discs under the same culture conditions as those used for dental pulp cells. The periodontal ligament cells had a flat, short fusiform, or fibroblast-like appearance. Cellular processes did not extend into the dentinal tubules and the cells did not form a multilayer (Fig. 5).

Fluorescence microscopy analysis of cytoskeleton architecture

Actin filaments were visualized by staining the cells with phalloidin. Figure 6 shows changes in the actin filaments over time. On day 1, labeling for actin, either on dentin discs or on slides, was homogeneously distributed throughout the cells (data not shown). On day 2, thick microfilaments along the long axis of the cytoplasm were evident on the dentin discs and stress fibers had begun to form (Fig. 6A). In the control group, labeling accumulated at the cell periphery, but stress fibers were not obvious (Fig. 6B). On day 4, stress fibers on the dentin discs were concentrated along the axis of the cytoplasm. In certain areas, stress fibers concentrated to form monopolar cytoplasmic processes. On day 10, parallel bundles of agglutinated stress fibers were observed throughout the entire cytoplasm (Fig. 6E). However, cells cultured on slides had a fibroblast-like or fusiform appearance and stress fibers were inconspicuous.

Proliferation of dental pulp cells in various culture environments

The dental pulp cells that were seeded onto the dentin discs proliferated minimally, in accord with previous reports (Huang et al., 2006b). Remarkably, the proliferative activity of cells that were seeded into mineral liquid was similar to that of cells seeded onto dentin discs. Cells in the culture medium group increased rapidly in number, exhibiting a logarithmic growth phase from day 3 to day 6, followed by a decrease in number and a plateau phase (Fig. 7). There was no significant difference in proliferative activity between the experimental group

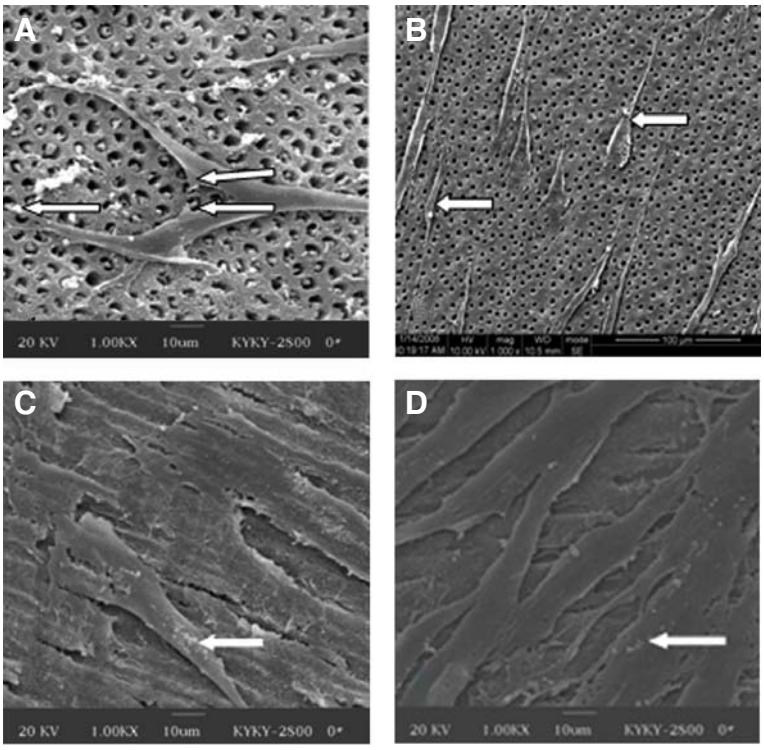

Fig. 4. Dental pulp cells at various times during culture on dentin discs. (A) Several endings of a process extending into dentinal tubules on day 2 of incubation (arrows). Bar = 10 μm. (B) Multiple cells formed a monopolar cytoplasmic process on day 2 (arrows). Bar = 100 μm. (C) Cell bodies were organized in parallel lines according to polarized cellular features on day 4 (arrow). Bar = 10 μm. (D) The punctate substance observed on the cell surfaces on day 10 may be secretory extracellular matrix (arrow). Bar = 10 μm.

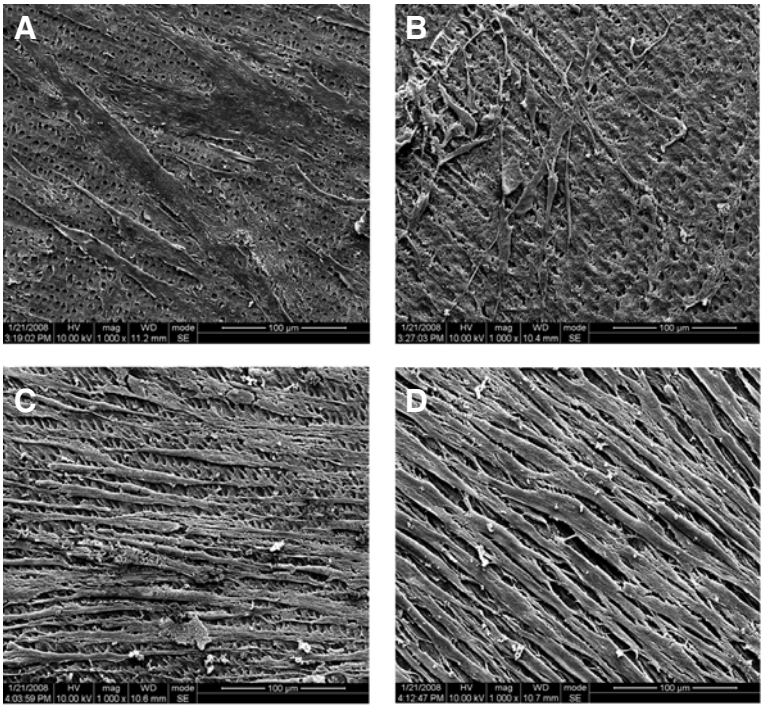

Fig. 5. Scanning electron microscopy images of periodontal ligament cells on dentin discs after culture for 1 day (A), 2 day (B), 4 day (C), and 10 day (D).

and the mineral liquid group (one-way ANOVA, $p > 0.05$), whereas proliferative activity was significantly higher for the culture medium group than for the experimental group ($p < 0.05$).

Expression of ALP under diverse culture conditions

ALP activity was used to study the cell differentiation properties of DPSCs because it promotes dentin calcification by reducing pyrophosphate level (Demer and Tintut, 2006). Figure 8 shows that an increase in ALP activity of dental pulp cells on dentin discs and of cells in mineral liquid medium was evident after 3 days of cultivation and was especially prominent on day 8 of cultivation. Thereafter, the ALP activity of the cells in the mineral liquid group reached a plateau, but that of the cells in the experimental group decreased. However, the expression level

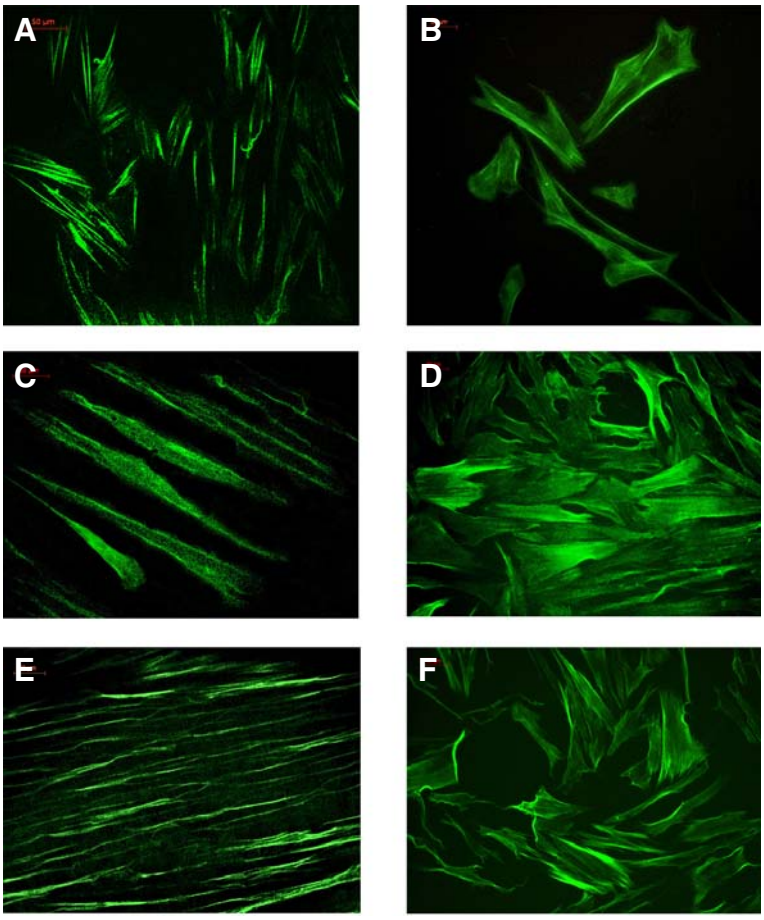

Fig. 6. Fluorescence microscopy images of actin filaments. (A, C, and E) Changes in the cytoskeleton of cells cultured on dentin discs were evident on days 2, 4, and 10. (B, D, and F) Phalloidin labeling of cells cultured on slides did not change with time.

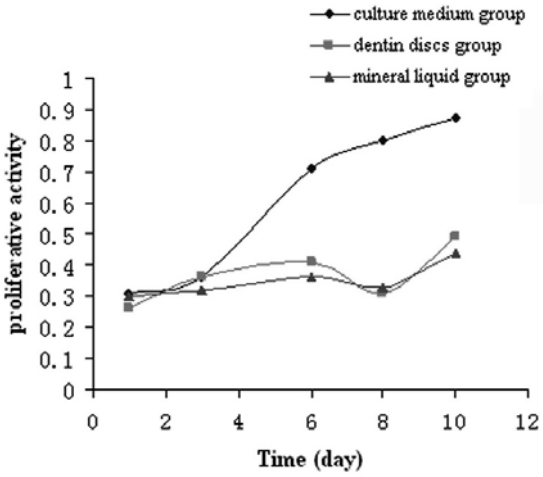

Fig. 7. MTT investigation of cell Proliferative activity under different conditions

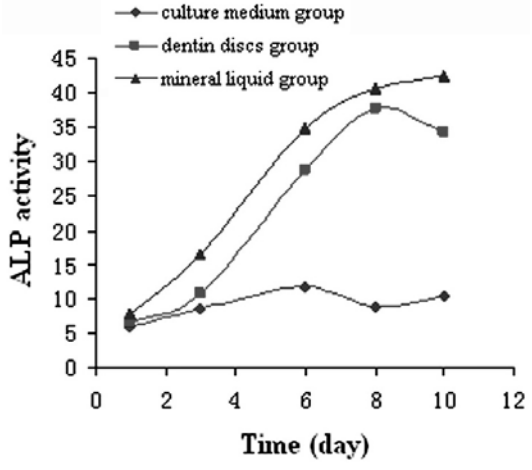

Fig. 8. The effect of various processing factors on the ALP activity of dental pulp cells.

of ALP in the medium group remained unchanged (Fig. 8). Overall, no significant difference in ALP activity was found between the experimental group and the mineral liquid group ($p > 0.05$). The ALP activities of the experimental and mineral liquid groups were much greater than that of the culture medium group ($p < 0.05$).

DISCUSSION

Considerable evidence suggests that resting progenitor or stem cells are present in the dental pulp (Gronthos et al., 2000; Suchánek et al., 2007). The ability of dental pulp cells to differentiate into odontoblast-like cells has long been recognized (Kim et al., 2009; Zhang et al., 2006). Several lines of evidence

have shown that odontoblast-like cells are capable of secreting a mineralized matrix to induce tertiary dentinogenesis (About et al., 2000; Nakashima and Akamine, 2005). However, there is no method for complete regeneration of odontoblasts. We studied the inductive role of the microenvironment using dentinal tubules with a natural three-dimensional structure, and successfully regenerated odontoblast-like cells.

Previous studies have shown that DPSCs show positivity for markers such as STRO-1, c-kit, CD34, CD146, and 3G5 (Shi et al., 2005; Yang et al., 2007), which are considered early markers of mesenchymal stem cells. In the present study, CD34-positivity indicated existence of stem cells among cultured dental pulp cells. After 2 days of culture on dentin discs, some, but not all, cells formed monopolar cytoplasmic processes. This phenomenon may reflect the conversion of DPSCs to a preliminary odontoblast morphology.

It is noteworthy that the end branches of some monopolar process extended into the dentinal tubules on day 2. Huang et al. (2006a) observed cell processes extending into dentinal tubules after 16 days of culture. This may be ascribed to further opening of the dentinal tubules. We observed multilayer formation on day 4, whereas immortalized bovine pulp cells grown on bovine dentin discs formed a multilayer after 14 days of culture. In our study, dental pulp cells seeded onto the dentin surface proliferated minimally, which is in accordance with the results of Huang et al. (2006b), but in disagreement with observations of cells planted on bovine dentin discs (Schmalz et al., 2001). This discrepancy may have been caused by differences in the donor species used to produce the dentin discs or the size of the aperture. Further opening of dentinal tubules may result in pore size enlargement, which could affect cell proliferation and differentiation.

Periodontal ligament cells seeded onto the dentin surface did not have the ability to differentiate into odontoblast-like cells. It seems that the dentin discs had a unique inductive effect on the dental pulp cells. However, the mechanism responsible for this effect remains to be elucidated.

The actin cytoskeleton is the principal determinant of cell shape, and cytoskeletal reorganization affects cell proliferation, differentiation, adhesion, and migration (Papakonstanti and Stournaras, 2008). F-actin is a fundamental structural unit of the cytoskeleton; therefore, we used an F-actin label to study the cytoskeleton. After 1 day of cultivation on dentin discs, labeling was homogeneously distributed throughout the cell, indicating that the cell had not responded to the dentin discs at that stage. On day 2, actin monomers bound together and formed stress fibers that were distributed along the long axis of the cytoplasm. The results are in accord with a previous report by Mesgouez (2006), which revealed that actin nucleation occurs to accommodate morphological changes. Actin nucleation provides a site for cell elongation (Prasain and Stevens, 2009). Consequently, stress fibers aggregated and were oriented in parallel on day 4. Significantly, some fibers aggregated to form a monopolar process, which corresponded with the SEM results.

In comparison with cells inoculated onto porous calcium phosphate (Wang et al., 2006), stress fibers on the dentin surface were more obvious and formed monopolar processes in certain areas. These differences can be explained as follows. Firstly, the density of cells growing on the limited surface area of dentin was high, which favors intercellular signaling. Secondly, the dentin surface demineralization exposed collagen-binding sites, which may have affected the differentiation of DPSCs. Thirdly, dentinal tubules may promote cytoskeleton reconstruction. Little information is available regarding the role of dentinal tubules. Further studies should explore this in the future.

Many studies have shown that ALP plays an essential role in the mineralization of dental tissues. ALP has been widely used as a phenotype marker of odontoblast-like cells *in vitro* (Schmalz et al., 2001; Wei et al., 2007). In the present study, levels of ALP activity for experimental group were much higher than those of the culture medium group, but were similar to those of the mineral liquid group. This shows that dentin discs can induce dental pulp cells to differentiate into odontoblast-like cells. We infer that the effect of dentin discs on cell differentiation is equal to that of mineral liquid. Whether dentin disc configuration (e.g., pore size) or composition (e.g., HA, collagen, or bioactive molecules) was primarily responsible for induction of cell differentiation remains unknown.

In conclusion, the dentin discs had an inductive effect on DPSCs that possessed some of the morphological and functional characteristics of odontoblasts. We observed that microfilaments recombined, cell proliferative activity decreased, and ALP activity increased. In particular, cells formed a multilayer and monopolar cytoplasmic processes that extended into dentinal tubules at an early stage of culture. We speculate that this phenomenon is associated with the open dentinal tubules. Thus, this method of treating dentinal tubules may facilitate advances in tissue engineering for dentinogenesis.

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