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Immortalization of human dental papilla, dental pulp, periodontal ligament cells and gingival fibroblasts by telomerase reverse transcriptase

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BACKGROUND: Human telomerase reverse transcriptase (hTERT) is catalytic subunit of human telomerase.
METHODS: We studied the immortalization of a series of human dental and periodontal cells by ectopic expression of hTERT and co-expression of hTERT with human papilloma virus 16 (HPV16) or simian virus 40 (SV40). Differentiation abilities of the established cell lines were studied in terms of the mineralized matrix formation and gene expression.
RESULTS: We established immortalized gingival fibroblasts by hTERT, dental papilla and periodontal ligament cells by hTERT and HPV16, and pulp cells by hTERT and SV40. The papilla and pulp cells showed mineralization and dentin sialophosphoprotein (DSPP) expression when cultured in the presence of β-glycerophosphate. The immortalized periodontal ligament cells did not show mineralization or DSPP expression, although expressions of alkaline phosphatase, osteopontin and osteocalcin were detected.
CONCLUSIONS: These cell lines will be useful tools for studying the repair and regeneration of dental and periodontal tissues and various diseases including odontogenic tumors.
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Keywords: dental papilla cell; dental pulp cell; dentin sialophosphoprotein; gingival fibroblast; immortalization; mineralization; periodontal ligament cell; telomerase reverse transcriptase

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Introduction

Immortalization of human normal cells has been reported by introduction of simian virus 40 (SV40) and human papilloma virus 16 (HPV16). In SV40, T-antigen, which inactivates the p53 and Rb proteins, has the ability of immortalization, while in HPV16, E6 and E7 proteins cooperatively immortalize cells. E6 promotes the degradation of p53 and E7 inactivates Rb protein (1). The cells expressing SV40 or HPV16 proliferate beyond the point at which their original cells become senescent, but most of the cells enter into a replicative senescent state referred to as crisis. Cells, which escape from the crisis, appear at a very low frequency and these cells acquire immortality.

Most immortalized cells and cancer cells show strong telomerase activity. Telomerase is a ribonucleo-protein complex that directs addition of the telomeric DNA repeats which undergo progressive shortening at each round of DNA replication in somatic cells (2, 3). Increased expression of human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, has been demonstrated to correlate with strong telomerase activities in cancer cells and immortalized cells (4–7). We also previously reported that the expression of hTERT correlates with telomerase activities in normal oral and ectocervical keratinocytes in serial passages, squamous cell carcinoma cells and SV40- and HPV16-immortalized cells (8, 9).

These results suggest that the ectopic expression of hTERT is able to immortalize cells, while co-expression of hTERT and HPV16 or SV40 should increase the efficiency of the immortalization. In this report, we studied the immortalization of human normal cells isolated from a series of dental and periodontal tissues by introducing hTERT and co-expression of hTERT with HPV16 or SV40 genes. We established immortalized gingival fibroblasts by hTERT, dental papilla cells

and periodontal ligament cells by hTERT and HPV16 and dental pulp cells by hTERT and SV40.

Materials and methods

Preparation of normal human cells

Under the approval of the Ethical Committee, School of Dentistry, University of Tokushima, extracted teeth and small pieces of oral mucosa were collected for isolation of normal cells at the time of tooth extraction for orthodontic treatments after obtaining informed consent. Gingival fibroblasts were primary cultured from a fragment of gingival mucosa excised at the extraction of an impacted tooth. Mucosal epidermis was removed and the remaining dermis was cut into small pieces and placed in culture dishes. Periodontal ligament was removed from the outermost surface at the middle third of tooth root of an erupted third molar with a scalpel. The cells were collected by centrifugation and seeded in dishes. Dental pulp was removed from the pulp cavity of an erupted third molar with a reamer after cutting of the tooth crown. The root pulp was cut into small pieces and placed in the culture dishes. For dental papilla cells, small tissue pieces were isolated from the central region of the tissue corresponding to dental papilla of an impacted incomplete third molar at the late bell stage. All the cells were cultured with α-MEM (Sigma Chemical Co., St Louis, MO, USA) containing 10% Fetal Calf Serum (Boehringer Mannheim, Tokyo, Japan) at humidified atmosphere of 5% CO_2 in air.

Transfection

The hTERT expression vector, pCI-Neo-hTERT (7) was a kind gift from Dr Weinberg (Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge, USA). Vectors of HPV16 (pSHPV16s) and SV40 (ori-) were previously described (10, 11). The cells at passage 3 were transfected with 1 μg of pCI-Neo-hTERT with or without 1 μg of the SV40 or HPV16 vectors using Tfx-20 (Promega, Tokyo, Japan) in 60 mm dishes. After 2 weeks of G418 selection (200 μg/ml), the resistant clones were isolated using a cloning ring (Iwaki, Tokyo, Japan) and maintained in serial culture without G418. The split ratio was 1:4 at early passages and 1:16 at later passages.

Detection of senescence-associated β-galactosidase

Cells were fixed in 2% formaldehyde/0.2% glutaraldehyde and stained with 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) in 40 mM citric acid/sodium phosphate, 5 mM potassium ferrocyanate, 5 mM potassium ferricyanate, 150 mM NaCl, 2 mM $MgCl_2$ (pH 6.4), for 12 h at 37°C as described by Dimri et al. (12).

Telomeric repeat amplification protocol assay

Telomeric repeat amplification protocol (TRAP) assay was carried out as described previously (8, 9). The reaction mixture was resolved by electrophoresis in 12% polyacrylamide gel. The gel was stained with ethidium bromide and observed using an ultraviolet transilluminator (FAS II; Toyobo, Osaka, Japan).

Cell proliferation

Cells (5×10^4) were plated on a 35-mm dish. The culture medium was changed every 2 days. Cells were trypsinized and the number of living cells was counted by the dye exclusion test using 0.15% trypan blue.

Tumorigenesis analysis

Cells were suspended at 5×10^6 cells in 300 μl phosphate-buffered saline (PBS) and subcutaneously inoculated into the dorsal area of severe combined immune deficiency (SCID) mice at 6 weeks of age (CLEA Japan Inc., Osaka, Japan). Three mice were used for each cell line. Observation was continued up to 16 weeks after inoculation.

Mineralized matrix formation

Confluent cells were cultured in the presence of 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate (Sigma) for 3 weeks. For the von Kossa staining, cells were fixed in 1% (w/v) paraformaldehyde in PBS and treated with 5% (w/v) silver nitrate (Sigma) in the dark for 15 min. Cells were rinsed with distilled water, incubated with 5% sodium thiosulfate (Sigma) for 2 min, rinsed again with distilled water and irradiated under a 100-W light for 5 min. For the quantification of mineralization, cells were fixed in 95% ethanol and stained with alizarin red S, then the calcium-bound dye was extracted with 1% HCl in 70% ethanol and the absorbance was measured at 450 nm using a microplate reader (Colona Electric Co. Ltd, Ibaragi, Japan).

Extraction of DNA and RNA and polymerase chain reaction analysis

Genomic DNA was extracted from cells using the proteinase K and phenol:chloroform method as described previously (9). Total RNA samples were purified from the cells using TRIZOL (Gibco BRL, Gaithersburg, MD, USA). RNA samples were treated with deoxyribonuclease I for 15 min at room temperature. The respective RNAs (2 μg) were converted into cDNA using random hexamer primers and reverse transcriptase (Gibco BRL) for 50 min at 42°C, followed by RNase H treatment for 20 min at 37°C. Polymerase chain reaction (PCR) with the reverse transcribed RNA (RT-PCR) or genomic DNA was carried out using PCR MASTER (Boeringer Mannheim) as described previously (8, 9). Amplified products were analyzed on 2% agarose gel. All of the primers used were obtained from Hokkaido System Science Co. Ltd. (Sapporo, Japan). The primer sequences and product sizes were: alkaline phosphatase (ALP), 475 bp, 5′-acgtggctaagaatgtcatc-3′ (forward), 5′-ctggtaggcgatgtcctta-3′ (reverse); type I collagen (ColI), 403 bp, 5′-tgacgagaccaagaactg-3′ (forward), 5′-ccatccaaaccactgaaacc-3′ (reverse); osteocalcin (OCN), 310 bp, 5′-catgagagccctcaca-3′ (forward), 5′-agagcga caccctagac-3′ (reverse); osteopontin (OPN), 347 bp, 5′-ccaagtaagtccaacgaaag-3′ (forward), 5′-ggtgatgtcctcg tctgta-3′ (reverse); dentin sialophosphoprotein (DSPP),

248 bp, 5′-gatgatcccaatagca-3′ (forward), 5′-cctttgccact gtctg-3′ (reverse); neomycin phosphotransferase, 155 bp, 5′-gattgcacgcaggttctccggc-3′ (forward), 5′-cagggcaccgga caggtcggtc-3′ (reverse); HPV16 E6, 472 bp, 5′-atgtt tcaggacccgc-3′ (forward), 5′-ttacagctgggtttct-3′ (reverse); HPV16 E7, 313 bp, 5′-atgcatggagatacac-3′ (forward), 5′-ttatggtttctgagaa-3′ (reverse); SV40 T antigen, 200 bp, 5′-catgaacagactgtgaggac-3′ (forward), 5′-tgtggctatggga attggag-3′ (reverse); glyceraldehyde-3′-phosphate dehy drogenase (G3PDH), 452 bp, 5′-accacagtccatgccatcac-3′ (forward), 5′-tccaccaccctgttgctgta-3′ (reverse).

Results

Establishment of immortalized gingival fibroblasts by hTERT transfection

Fibroblasts from the fragments of gingival dermis showed outgrowth as an explant culture. At the third passage, cells were transfected with an hTERT expression vector or the control vector (pCI-Neo). Each of three clones were isolated after G418 selection. The parental fibroblasts and the control vector-transfected clones ceased growth at about passage 25. Two of the hTERT-transfected clones ceased propagation at about passage 30. However, one hTERT-transfected clone showed a continuous growth over 150 population doubling levels (PDL). This gingival fibroblast clone was designated as GT-1. The expression of senescence-associated β-galactosidase, a marker of aging in cultured normal cells (12), was studied in the parental fibroblasts and GT-1 cells (Fig. 1). The gingival fibroblasts (at passage 20) but not GT-1 cells (at passage 40) showed expression of β-galactosidase, suggesting that the fibroblasts were in the state of senescence while GT-1 cells had passed the senescence and acquired immortality.

Immortalization of dental papilla, dental pulp, and periodontal ligament cells

Cells from dental papilla, dental pulp and periodontal ligament showed a similarly spindle-shaped, fibroblastic morphology. At the third passage, the hTERT expression vector was transfected into these cells with or without SV40 or HPV16 vector. Unlike gingival fibroblasts, no immortalized cells were obtained by transfection of hTERT. However, a clone of dental papilla cells transfected with hTERT and HPV16, a clone of dental pulp cells transfected with hTERT and SV40 and a clone of periodontal ligament cells transfected with hTERT and HPV16 showed continuous growth over 150 PDL. We named these immortalized clones of dental papilla, dental pulp, and periodontal ligament cells PA-1, DP-1 and PLT-1, respectively.

Telomerase activities in these cells were analyzed by TRAP assay (Fig. 2). GT-1 (lane 2), PLT-1 (lane 4), DP-1 (lane 6), and PA-1 cells (lane 8) at passage 40 showed 6 bp DNA ladders which were absent in the samples of their parental cells at passage 3 (lanes 1, 3, 5, and 7). These ladders were not observed in the heat-treated samples (data not shown), indicating the specific detection of telomerase activities in these immortalized cells as previously reported (8).

The integration of transfected genes in these cells was confirmed by PCR using the cellular DNA (Fig. 3). Neomycin phosphotransferase is present in the hTERT

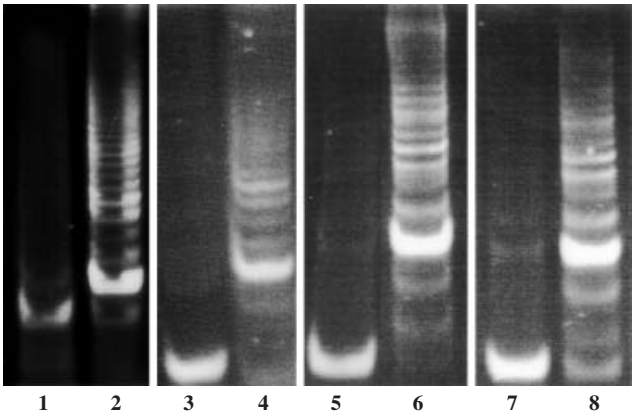

Figure 2 Telomerase activities in normal and immortalized cells. Telomerase activity was analyzed by telomeric repeat amplification protocol (TRAP) assay. Lane 1, gingival fibroblasts; lane 2, immortalized gingival fibroblasts (GT-1); lane 3, periodontal ligament cells; lane 4, immortalized periodontal ligament cells (PLT-1); lane 5, dental pulp cells; lane 6, immortalized dental pulp cells (DP-1); lane 7, dental papilla cells; lane 8, immortalized dental papilla cells (PA-1).

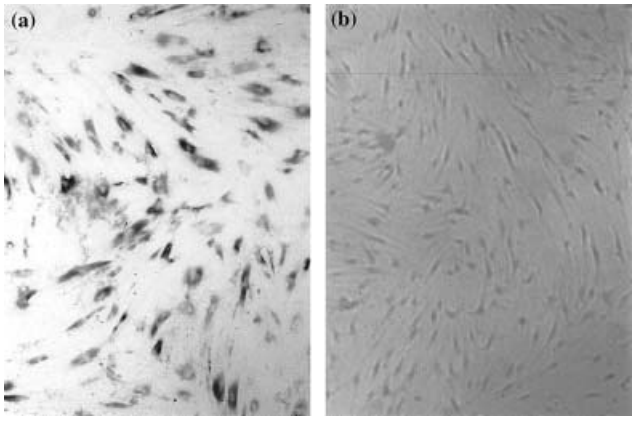

Figure 1 Expression of senescence-associated β-galactosidase in normal gingival fibroblasts at passage 20 (a) and the immortalized gingival fibroblasts at passage 40 (b).

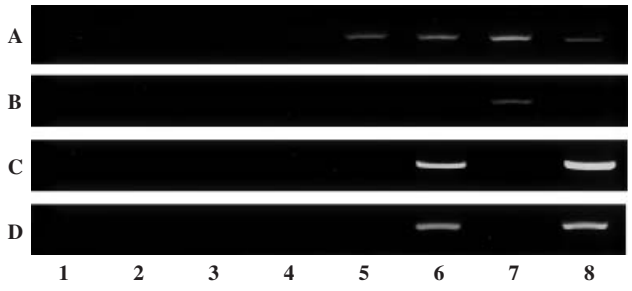

Figure 3 Integration of transfected genes in immortalized cells. DNA was isolated from the cells and analyzed by polymerase chain reaction (PCR). (a) Neomycin phosphotransferase, (b) SV40 T-antigen, (c) HPV16 E6, (d) HPV16 E7. Lane 1, gingival fibroblasts; lane 2, periodontal ligament cells; lane 3, dental pulp cells; lane 4, dental papilla cells; lane 5, immortalized gingival fibroblasts (GT-1); lane 6, immortalized periodontal ligament cells (PLT-1); lane 7, immortalized dental pulp cells (DP-1); lane 8, immortalized dental papilla cells (PA-1).

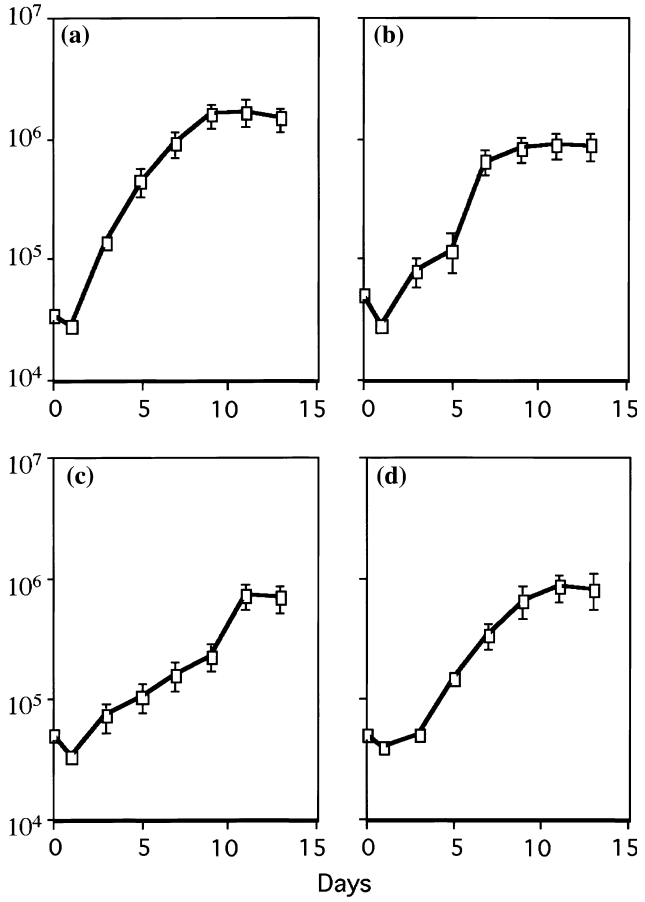

Figure 4 Growth curves of immortalized cells at passage 40. Each point shows the mean and SD of viable cells counted in triplicate. (a) immortalized dental papilla cells (PA-1), (b) immortalized dental pulp cells (DP-1), (c) immortalized periodontal ligament cells (PLT-1), (d) immortalized gingival fibroblasts (GT-1).

expression vector and was detected in GT-1, PLT-1, DP-1, and PA-1 cells but not in their parental cells. SV40 T-antigen was detected in DP-1 cells but not in other cells. Similarly, the PCR products of HPV16 E6 and E7 were detected in PLT-1 and PA-1 cells but not in other cells. The growth curves of these cells at passage 40 are shown in Fig. 4. The doubling times of PA-1, DP-1, PLT-1, and GT-1 were 28.9, 32.0, 54.3 and 38.4, respectively. These cells showed no tumor formation when inoculated subcutaneously into SCID mice (data not shown).

Characterization of immortalized dental papilla, dental pulp, periodontal ligament cells and gingival fibroblasts

These immortalized cells showed a similar spindle morphology in the growth phase (Fig. 5a–d). After confluence (Fig. 5e–h), cells were cultured for 3 more weeks in the presence of 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate, and their activities of mineralized matrix formation were examined. PA-1 (Fig. 5i) and DP-1 cells (Fig. 5j) but not GT-1 (Fig. 5k) or PLT-1 cells (Fig. 5l) showed mineralization which was detected by the von Kossa staining. The levels of mineralization in these cells were compared by measuring the absorbance of the extracted dye from the cells after staining with alizarin red S (Fig. 6). PA-1 cells (Fig. 5a) showed a strong activity of mineralization, while DP-1 cells (Fig. 5b) showed a weak activity, and no mineralization was detected in GT-1 (Fig. 5c) or PLT-1 cells (Fig. 5d).

We further studied the gene expressions of ColI, ALP, OPN, OCN, and DSPP in these cells (Fig. 7). RNAs were isolated from the confluent cells cultured for 3 weeks in the presence of ascorbic acid and β-glycerophosphate. RT-PCR analysis revealed that all four cell lines showed a similar level of ColI expression. Expression of ALP was observed strongly in PA-1 (lane 1) and DP-1 (lane 2), weakly in PLT-1 (lane 3) but not in GT-1 cells (lane 4). Similarly, expression of OPN was strong in PA-1 and DP-1, weak in PLT-1 but not observed in GT-1 cells. OCN was expressed in PA-1, DP-1, and PLT-1 but not in GT-1 cells. DSPP was strongly expressed in PA-1 cells. DP-1 cells also showed expression at a lower degree, however, no expression was observed in GT-1 or PLT-1 cells.

Discussion

In this study, we established human immortalized cell lines of dental papilla, dental pulp, periodontal ligament and gingival fibroblasts. The gingival fibroblast cell line, GT-1, was established by introduction of hTERT cDNA. The periodontal ligament cell line, PLT-1, and the dental papilla cell line, PA-1, were obtained by gene transferring of hTERT and HPV16. The dental pulp cell line, DP-1, was immortalized by hTERT and SV40. These cells showed a strong telomerase activity and no tumor formation in SCID mice.

Several previous reports suggested that the ability of hTERT to immortalize human cells is dependent on the cell type. The immortalization of human fibroblasts by introduction of hTERT cDNA has been reported (13). Human bone marrow mesenchymal cells, which could differentiate into osteoblasts and chondrocytes, have also been immortalized by ectopic expression of hTERT (14). However, both SV40 T-antigen and hTERT were required for the immortalization of human adult osteoblasts (15). Similarly, both induction of telomerase and inactivation of pRb/p16INK4a were required for immortalization of human skin keratinocytes (16, 17). Consistent with these reports, we previously reported the establishment of immortalized human oral keratinocytes by transfection with expression vectors of hTERT and HPV16 E7 (18). Although further studies are needed to clarify whether or not the expression of hTERT is sufficient for immortalization of these human cells, our results indicated that co-expression of hTERT with other immortalizing genes, SV40 T-antigen or E6 and E7 of HPV16, increases the efficiency of immortalization of these cells.

Human dental papilla cells have been reported to differentiate into odontoblasts in culture (19). Similar phenotypes of dental pulp cells have also been reported (20–23). They are characterized by the expression of proteins, which are common in mineralized tissues, and tooth-specific proteins, such as DSPP gene products (dentin sialoprotein and dentin phosphoprotein) (24–26).

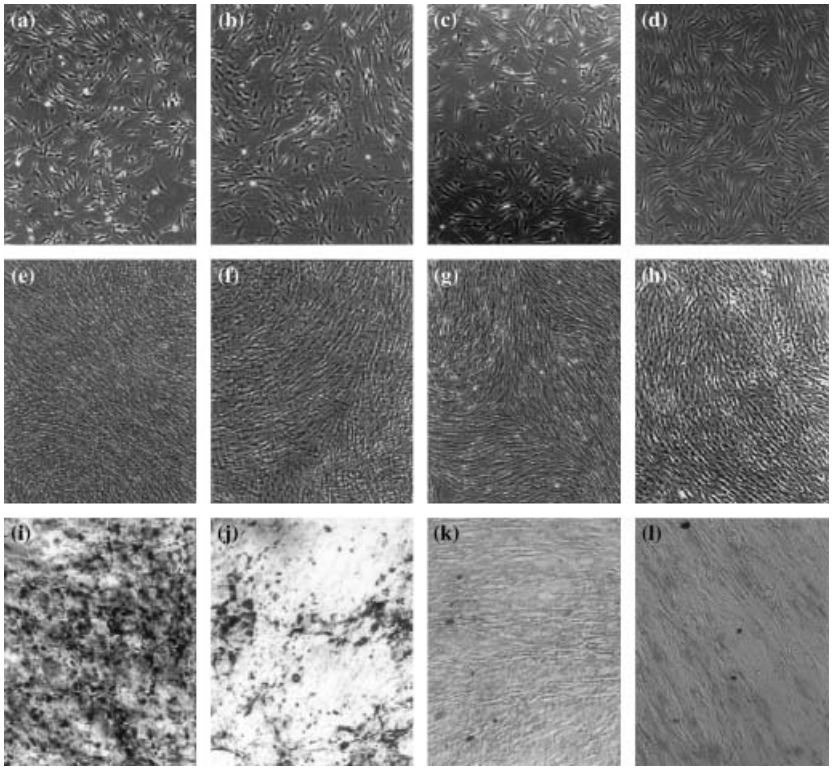

Figure 5 Morphology and mineralized matrix formation of immortalized cells. Photographs of cells at growing phase (a–d) and confluence (e–h) were taken under a phase-contrast microscope at a magnification of ×100. Mineralized matrix formation (i–l) was analyzed by von Kossa staining. Cells cultured in the presence of ascorbic acid and β-glycerophosphate for 3 weeks. (a, e, i) immortalized dental papilla cells (PA-1), (b, f, j) immortalized dental pulp cells (DP-1), (c, g, k) immortalized periodontal ligament cells (PLT-1), (d, h, l) immortalized gingival fibroblasts (GT-1).

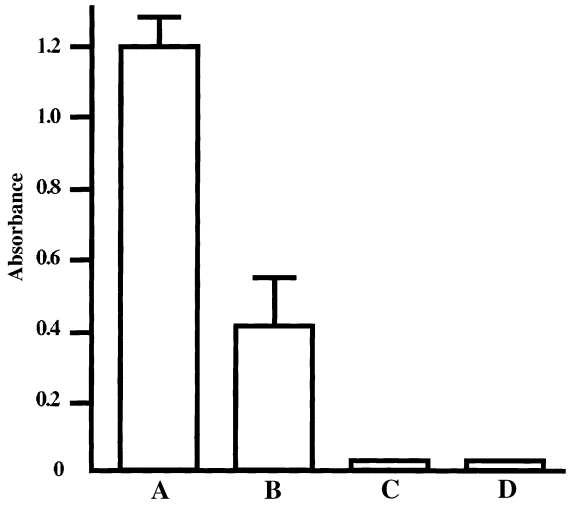

Figure 6 Activities of mineralized matrix formation. Cells were cultured in the presence of ascorbic acid and β-glycerophosphate for 3 weeks and stained with alizarin red S, then the dye was extracted and the absorbance at 450 nm was measured. (A) immortalized dental papilla cells (PA-1), (B) immortalized dental pulp cells (DP-1), (C) immortalized periodontal ligament cells (PLT-1), (D) immortalized gingival fibroblasts (GT-1).

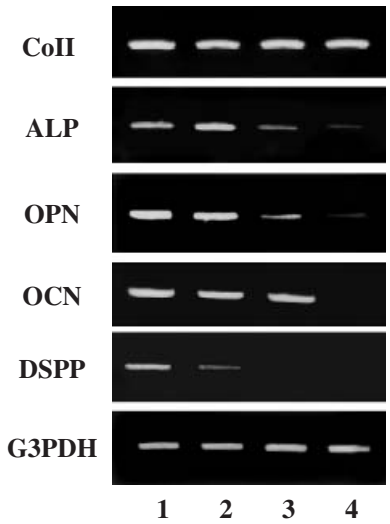

Figure 7 Gene expressions in immortalized cells. Cells were cultured in the presence of ascorbic acid and β-glycerophosphate. RNA samples were isolated and analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). Lane 1, immortalized dental papilla cells (PA-1); Lane 2, immortalized dental pulp cells (DP-1); Lane 3, immortalized periodontal ligament cells (PLT-1); Lane 4, immortalized gingival fibroblasts (GT-1).

In the present study, both PA-1 and DP-1 cells showed mineralized matrix formation and expression of ALP, OPN, OCN, and DSPP genes when cultured in the presence of β-glycerophosphate, indicating that they have abilities of differentiation into odontoblasts, although the odontoblast-phenotype of DP-1 cells was less prominent than that of PA-1 cells.

In contrast, PLT-1 and GT-1 cells showed neither expression of DSPP nor mineralization when cultured in the same condition, although expressions of ALP, OPN, and OCN were observed in PLT-1 cells. The periodontal ligament is located between the cementum of teeth and the alveolar bone and is kept free of mineralization. The predominant cells of periodontal ligament have a fibroblastic morphology, no or very low activity of calcified nodule formation, but different gene expressions from gingival fibroblasts in culture (27–29). The present results, taken together with these previous reports, indicated that PLT-1 cells retain the normal phenotype of periodontal ligament cells.

Establishments of immortalized cells with odontoblast-phenotypes have been reported from dental papilla of bovine (30, 31), mouse (32) and rat (33), and from dental pulp of rat (34). Immortalized periodontal ligament cells from mice (29, 35) and humans (36) have been also reported. However, there has been no report of immortalization of human cells derived from a series of dental and periodontal tissues. The cell lines established in the present study showed stable growth abilities and the normal phenotypes. These cells will be useful tools for the study of biological characterization, repair and regeneration of human dental and periodontal tissues and the diagnosis and mechanisms of various diseases including odontogenic tumors in the oral region.

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