J Periodont Res 2007; 42; 104–113

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2006.00921.x

Differential expression of periodontal ligament-specific markers and osteogenic differentiation in human papilloma virus 16-immortalized human gingival fibroblasts and periodontal ligament cells

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Pi S-H, Lee S-K, Hwang Y-S, Choi M-G, Lee S-K, Kim E-C. Differential expression of periodontal ligament-specific markers and osteogenic differentiation in human papilloma virus 16-immortalized human gingival fibroblasts and periodontal ligament cells. J Periodont Res 2007; 42: 104–113. © 2006 The Authors. Issue compilation © 2006 Blackwell Munksgaard

Background and Objective: Periodontal ligament cells and gingival fibroblasts are important in the remodeling of periodontal tissue, but human papilloma virus (HPV)16-immortalized cell lines derived from human periodontal ligament cells and gingival fibroblasts has not been characterized. The purpose of this study was to establish and differentially characterize the immortalized cell lines from gingival fibroblasts and periodontal ligament by HPV16 transfection.

Material and Methods: Cell growth, cell cycle analysis, western blot for cell cycle regulatory proteins and osteogenic differentiation markers, and reverse transcription–polymerase chain reaction for periodontal ligament-specific markers were performed.

Results: Both immortalized cell lines (immortalized gingival fibroblasts and immortalized periodontal ligament cells) grew faster than primary cultured gingival fibroblasts or periodontal ligament cells. Immortalized gingival fibroblasts and immortalized periodontal ligament cells overexpressed proteins p16 and p21, and exhibited degradation of proteins pRb and p53, which normally cause cell cycle arrest in G_2/M-phase. Western blotting and reverse transcription–polymerase chain reaction for periodontal ligament-specific and osteogenic differentiation marker studies demonstrated that a cell line, designated IPDL, mimicked periodontal ligament gene expression for alkaline phosphatase, osteonectin, osteopontin, bone sialoprotein, bone morphogenic protein-2, periostin, S-100A4 and *PDLs17*.

Conclusion: These results indicate that IPDL and immortalized gingival fibroblast cell lines consistently retain normal periodontal ligament and gingival fibroblast phenotypes, respectively, and periodontal ligament markers and osteogenic differentiation in IPDL are distinct from immortalized gingival fibroblast cells.

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Key words: cell line; gingival fibroblasts; osteogenic differentiation; periodontal ligament

Accepted for publication May 29, 2006

Gingival fibroblast cells and periodontal ligament cells are the major cellular components of periodontal connective tissue, and these cells are responsible for the overall production and turnover of the extracellular matrix of periodontal tissue (1,2).

Periodontal ligament cells and gingival fibroblast culture systems are important tools for the investigation of periodontal metabolism, which is essential for identifying the pathways of normal and pathological degeneration and tissue inflammation (1). Several studies have demonstrated that different heterogenic properties exist in cell cultures derived from human periodontal ligament, with cells exhibiting both fibroblast-like and osteoblast-like characteristics (3). Periodontal ligament cells may also have osteoblastic phenotypes, such as high alkaline phosphatase (ALPase) activity, parathyroid hormone responsiveness, production of bone-like matrix proteins and formation of mineralized nodules (4–7). Phenotypic variation in periodontal ligament cell cultures can occur with increasing passage numbers, and cell lines derived from different patients may exhibit considerable variability (8,9).

In recent years, S100 calcium-binding protein and periostin (osteoblast-specific factor-2, osteogenic supplements F2) have been identified as phenotypic markers that distinguish periodontal ligament and gingival fibroblast cells (10). S100A4 is also postulated to play a role as a mineralization inhibitor that is responsible for maintaining the extracellular periodontal ligament space (11). Periostin, which was originally isolated from osteoblast cell lines (e.g. MC3T3E1), plays a specific role in the formation of mineralized tissue (12). Periostin is preferentially expressed in the periosteum and periodontal ligament, indicating its tissue specificity and potential role in bone and tooth formation and structure maintenance. The novel gene, PDLs17, is highly expressed in periodontal ligament, bone marrow and osteoblast cells, but not in gingival fibroblasts (13). Therefore, S100A4, periostin and PDLs17 may participate in the regeneration and/or differentiation of periodontal ligament cells, for which they can be used as specific markers.

Current periodontal research is heavily dependent on immortalized periodontal ligament and gingival fibroblast cell lines, which provide permanent and abundant sources of cells representing the differentiated PDL and gingival fibroblast phenotypes. Although several immortalized periodontal ligament cell lines of animal or human origin have been developed using simian virus 40 (SV40) and telomerase reverse transcriptase (14–20), immortalization of periodontal ligament and gingival fibroblast cells using the human papilloma virus (HPV)16 E6/E7 oncogene has not been previously reported. Moreover, little is known about comparative studies on immortalized gingival fibroblasts conpared with periodontal ligament cells.

In this study, we established immortalized human periodontal ligament and gingival fibroblast cell lines for further use in fundamental and clinical periodontal research by transfecting the cells with an HPV16 E6/E7 oncogene construct. The resulting immortalized cell lines were characterized by growth, morphology, cell cycles analyses, ALPase enzyme assays, Alizarin red staining, and western blotting and reverse transcription–polymerase chain reaction (RT–PCR) studies of expression of osteogenic differentiation markers, cell cycle regulatory proteins and periodontal ligament markers, such as S100A4, *PDLs17* and periostin.

Material and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and other tissue culture reagents were purchased from Gibco BRL (Grand Island, NY, USA). Anti-p16, -p21, -p53 and -pRb immunoglobulin were purchased from Santa Cruz (Santa Cruz, CA, USA). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Primary culture of periodontal ligament and gingival fibroblasts

Human periodontal ligament and gingival fibroblasts were isolated with an explant culture technique, from patients undergoing orthodontic treatment, using previously described methods (21). Informed consent from donors was obtained for use of the tissues. Patients signed the corresponding informed consent approved by the Institutional Review Board at Wonkwang University for use of the tissues. Briefly, these tissues were cut into 1 mm^2 explants and placed on 60 mm culture dishes (Nunc, Naperville, IL, USA) containing 10,000 U/ml of penicillin G sodium, 100 μg/ml of streptomycin sulphate, 25 μg/ml of amphotericin B, and 10% heat-inactivated fetal bovine serum (Gibco BRL) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 2 or 3 d, the cells started to outgrow from the explants. When the primary cell culture reached confluence, the cells were detached with 0.025% trypsin and 0.05% EDTA, diluted with culture medium and then subcultured in a ratio of 1 : 4. Cell cultures between the 3rd and 6th passage were used in this study.

Immortalization

HPV-immortalized human periodontal ligament (IPDL) and -gingival fibroblasts were derived by transfecting normal human gingival fibroblasts and periodontal ligament cells with PLXSN vector containing the E6/E7 open reading frames of HPV type 16, following the methods previously described (22). Stably transfected cells were selected using G418. The IPDL and immortalized gingival fibroblast were cultured in DMEM (Gibco BRL) containing 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 U/ml streptomycin (Life Technologies, Gaithersburg, MD). All the cell lines were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were dissociated with 0.25% trypsin just before transfer for experiments and were counted using a hemocytometer.

Assay for telomerase activity

Telomeric activity was measured using the $TRAP^{®}_{EZE}$ Telomerase Detection Kit (Chemicon® International, Temecula, CA, USA), as described previously (23). Cell extracts (1 μg of protein) were added to telomerase extension reactions and incubated for 30 min at 30°C. Polymerase chain reaction (PCR) was performed using the TS primer and PR primer for 33 cycles (denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min). As an internal teomerase assay standard, K1 primer and TSK1 template were added to the PCR mixture. Telomerase products were resolved by exlectrophoresis on a 12.5% nondenaturing polyacrylamide gel. Bands were then visualized by staining with ethidium bromide (Sigma). Relative telomerase activities were quantitified by comparing signal intensities among the lanes and with the positive control.

Cell proliferation assay

Cell proliferation was determined by viable cell counting**.** For cell number counting, the cells were cultured at 1×10^4 cells per well in a six-well culture plate. The number of viable cells after Trypan blue exclusion was counted after 1–10 d of incubation at 37°C. Triple cultures were simultaneously performed at each time.

Cell cycle analysis

The cultured cells were harvested by trypsinization and washed with phosphate-buffered saline (PBS). The cells were then fixed in cold 70% ethanol for 45 min at 4°C, pelleted, resuspended in TSP solution (0.1% Triton X-100, 0.1% sodium citrate and 0.005% propidium iodine) containing 1 μg/ml RNAse A and incubated for 30 min at room temperature (21–23°C). The state of the cell cycle was then analyzed on FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). Triple experiments were simultaneously carried out at each time.

ALPase assay

After the cells were plated in six-well plates at 1×10^5 cells/well, they were cultured in DMEM containing 10% fetal bovine serum, with or without osteogenic supplements (50 μg/ml ascorbic acid, 10 mM sodium α-glycerophosphate and 0.1 μM dexamethasone), and normal saline was added in the negative control group. Each group was then incubated for 14 d. The cells were separated by trypsin/EDTA and centrifuged at 20,000 ***g*** for 10 s. An aliquot was removed and centrifuged again at 20,000 ***g*** for 10 s. Another aliquot was removed and suspended in 0.5 ml of sterilized distilled water. Each 0.1 ml of suspension was mixed with 0.2 ml of 0.1 M glycine NaOH buffer (pH 10.4) containing 0.1 ml of 15 mM *para*-nitrophenyl phosphate (Sigma), 0.1 ml of 0.1% Triton X-100/saline and 0.1 ml of sterilized distilled water. The culture dishes were incubated at 37°C for 30 min, and the reaction was stopped by the addition of 0.6 ml of 0.1 N NaOH. The cultured cells were transported on 96-well plates and the absorbance was measured at 410 nm in an enzyme-linked immunosorbent assay (ELISA) reader. The standard concentrations of protein were calculated using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA). The results were expressed as nmol of *para*-nitrophenol released per min/mg of protein.

In vitro mineralization assay

Mineralization was assessed by staining with Alizarin red (Sigma-Aldrich, St Louis, MO, USA), according to the methods previously described (24). Briefly, 40 mM Alizarin red was prepared in distilled water, adjusted to pH 5.5 with ammonium hydroxide, and then applied to the cells in 12- to 24-well plates for 30 min at room temperature with gentle agitation. The cells were then washed with distilled water and allowed to dry before mineralization was documented using a Nikon TE200 Eclipse inverted microscope equipped with a SPOT™ digital camera (Diagnostics Instruments, Inc., Sterling Heights, MI, USA).

Western blot analysis

Cells grown in 100 mm Petri dishes were lysed with lysis buffer [10 mM Na_2HPO_4 (pH 7.2), 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.2% sodium azide and 0.004% sodium fluoride] on ice for 15 min. Cell lysates were centrifuged at 15,000 *g* for 5 min at 4°C, and the supernatant (100 mg/each lane) was mixed with an equal volume of 2 × sodium dodecyl sulfate sample buffer, boiled for 5 min, and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through 12–15% gels. After electrophoresis, proteins were transferred to nylon membranes by electrophoretic transfer. The membranes were blocked for 1 h in 5% skim milk, rinsed, and incubated with primary antibodies for p16, p21, p53, pRb, osteopontin (OPN), osteonectin (ON), bone sialoprotein (BSP), and bone morphogenic protein-2 (BMP) in phosphate buffered saline Tween-20 overnight at 4°C. Excess primary antibody was then removed by washing the membranes four times in phosphate buffered saline Tween-20, and the membrane was incubated with 0.1 μg/ml peroxidase-labeled secondary antibodies (against rabbit) for 1 h. After three washes in phosphate buffered saline Tween-20, bands were visualized by enhanced chemiluminescence and exposed to X-ray film.

RNA isolation and RT–PCR

The cells plated in 60-mm culture dishes were incubated for 14 d in a fresh medium, with or without osteogenic supplements. After discarding growth medium, total RNA was isolated from cells using easy-Blue (iNtRON Biotechnology, Daejon, Korea), following the manufacturer's instructions. Reverse transcription of the RNA was performed using AccuPower RT PreMix (Bioneer, Daejon, Korea). One microgram of RNA and 20 pmol primers were preincubated at 70°C for 5 min and transferred to a mixture tube. The reaction volume was 20 μl. cDNA synthesis was performed at 42°C for 60 min, followed by reverse

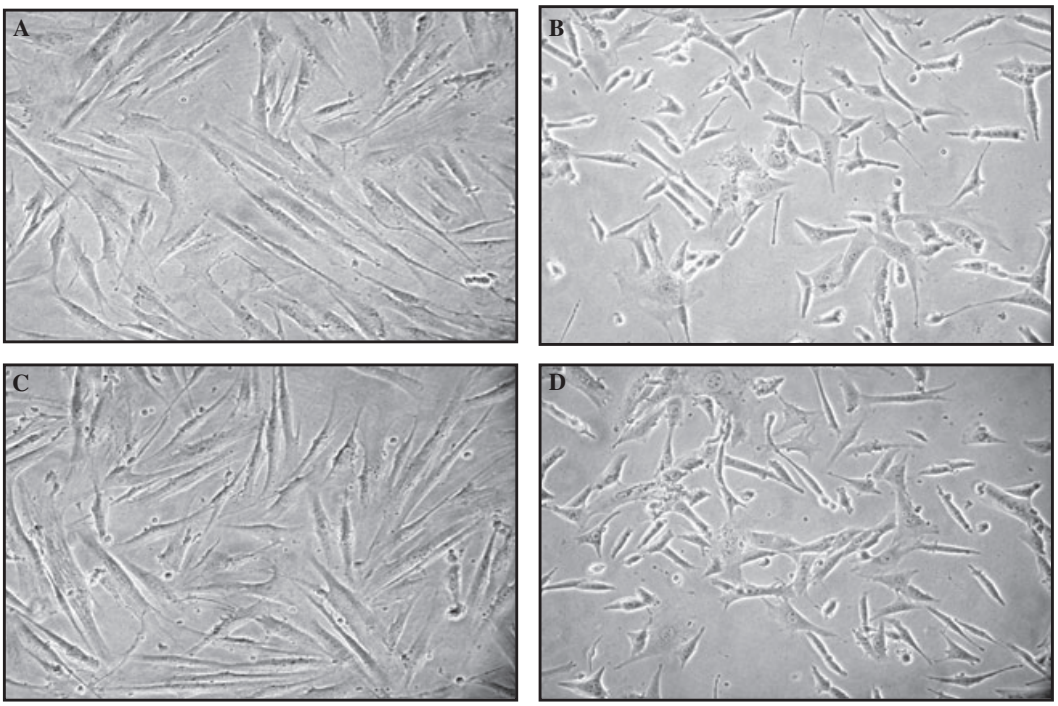

Fig. 1. Phase-contrast microphotography for gingival fibroblasts (GF) (A), immortalized gingival fibroblasts (IGF) (B), periodontal ligament cells (PDL) (C) and immortalized periodontal ligament cells (IPDL) (D), at the 60th passage.

transcription inactivation at 94°C for 5 min. Thereafter, the reverse transcription-generated DNA (2–5 μg) was amplified using AccuPower PCR PreMix (Bioneer). The primers used for cDNA amplification and PCR conditions were as follows: HPV16 E6, 472 bp (F) 5′-ATGTTTCAGGACCCGC-3′, (R) 5′-TTACAGCTGGGTTTCT-3′; HPV16 E7, 313 bp, (F) 5′-ATGCATGGAGATACAC-3′, (R) 5′-TTATGGTTTCTGAGAA-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 452 bp, (F) 5′-ACCACAGTCCATGCCATCAC-3′, (R) 5′-TCCACCACCCTGTT GCTGTA-3′; S100A4, 295 bp, (F) 5′-GGCCCTGGATGTGATGGTGT-5′, (R) 5′-TCCACCACCCTGTTGCTGTA-3′; periostin, 650 bp, (F) 5′-CACACTCTTT GCTCCCACC-3′, (R) 5′-GAATCGCACCGTTTCTCC-3′; PDLs17, 450 bp, (F) 5′-ATGGAACTATTATTATTAGAAG-3′, (R) 5′-ACCTTTCAAAACATGGAGTAA-3′.

The PCR conditions was performed annealing temperature of 55°C (periostin, S100A4 13,25), 56°C (GAPDH), 60°C (PDLs17), and 62°C (HPV16 E6/E7). After 25 (Periostin) and 30 (PDLs17, S100A4, GAPDH, HPV16 E6/E7) cycles (13), aliquots of the PCR products were resolved on 0.5% tric-acetate-EDTA-agarose gels, stained with ethidium bromide and photographed under ultraviolet light.

Statistical analysis

Values were calculated as the mean and standard deviation. Statistical significance was evaluated by one way analysis of variance (ANOVA) using the SPSS (v10.0; SPSS, Chicago, IL, USA) computer program.

Results

Cell morphology and growth

The periodontal ligament and gingival fibroblast cells used in this study were indistinguishable by light microscopy (Fig. 1). They exhibited typical spindle-shaped fibroblast morphologies. At the third passage of the fibroblasts obtained from gingival fibroblasts and periodontal ligament cells, the cells were transfected with an HPV16 E6/E7 expression vector or a control vector (PLXSN-Neo). Four transfected gingival fibroblast clones and 18 transfected polyvinylidense fluoride clones were then isolated using G418 selection. However, only one gingival fibroblast clone and two periodontal ligament clones displayed continuous growth past the 40th passage. These immortalized clones of gingival fibroblasts and periodontal ligament cells were designated IGF, IPDL15 and IPDL17, respectively. No morphologic differences between the IGF and IPDL cell lines were observed. Like primary cultured cells, the IGF and IPDL cells are also spindle-shaped at the 60th passage (Fig. 1).

The growth rate of these cells has remained reasonably constant since immortalization, requiring subculture once a week at a splitting ratio of 3 : 4. They exhibited rapid growth and higher proliferation than primary cells at the 60th passage (Fig. 2).

Cell cycle analysis

To determine whether HPV16-induced growth stimulation of gingival fibroblasts and periodontal ligament cells is

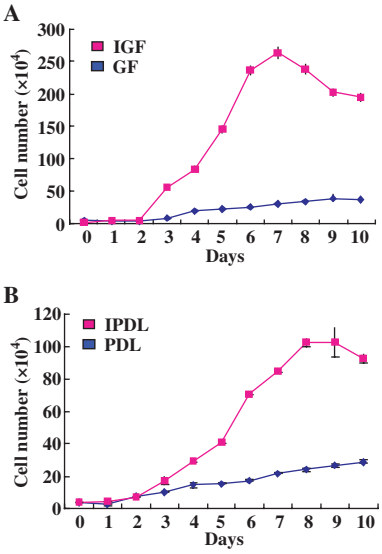

Fig. 2. Growth curves of primary cultured gingival fibroblasts (GF) and immortalized gingival fibroblasts (IGF) (A), periodontal ligament cells (PDL) at the 5th passage and immortalized periodontal ligament cells (IPDL) at the 60th passage (B). Each point shows the mean and standard deviation (SD) of viable cells counted in triplicate.

Table 1. Change of cell cycle, as determined by flow cytometry, in primary cultured vs. immortalized gingival fibroblasts and periodontal ligament cells

	G_1 (%)	S (%)	G_2/M (%)
GF	75.44 ± 0.49	6.91 ± 0.08	15.49 ± 0.30
IGF	71.78 ± 0.67	5.85 ± 0.92	17.72 ± 0.30
PDL	84.78 ± 0.67	6.17 ± 0.15	8.31 ± 0.28
IPDL	81.76 ± 1.17	2.06 ± 0.22	11.28 ± 0.83

These data are the mean ± standard deviation of three independent experiments.
GF, gingival fibroblasts; IGF, immortalized gingival fibroblasts; IPDL, immortalized periodontal ligament cells; PDL, periodontal ligament cells.

a result of alterations in the cell cycle, their cellular DNA was analyzed by FACScan using propidium iodide detection. In IGF and IPDL cells at the 60th passage, the HPV16 E6/E7 oncogene appeared to enhance cell cycle progression by increasing the G_2/M-phase ratio, whereas the G_2/M-phase ratio in gingival fibroblasts and periodontal ligament cells was relatively low (Table 1).

Confirmation of HPV16 E6/E7 integration

The presence of the HPV16 E6/E7 oncogene in transfected cells was examined by PCR. Electrophoretic analysis of the PCR amplification products demonstrated cellular integration of HPV16 E6/E7 in all transfected cells at the 60th passage (Fig. 3). Amplification products of appropriate size were detected in all IGF and IPDL cells, whereas no corresponding DNA band was detected in wild-type gingival fibroblasts or periodontal ligament cells.

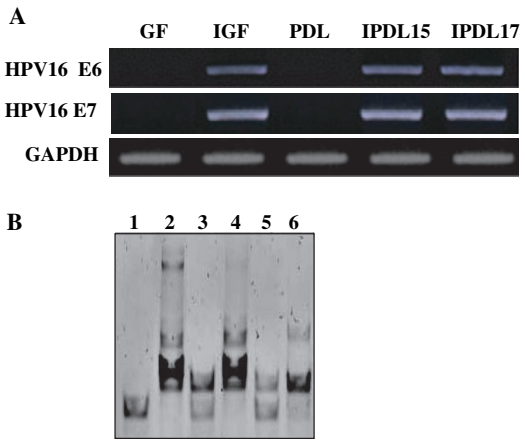

Fig. 3. (A) Gel electrophoretic analysis of polymerase chain reaction (PCR) amplication products of human papillomavirus (HPV)16 E6/E7-specific primer. Lane 1, gingival fibroblasts (GF); lane 2, immortalized gingival fibroblasts (IGF); lane 3, periodontal ligament cells (PDL); lane 4, immortalized periodontal ligament cells (IPDL15); lane 5, immortalized periodontal ligament cells (IPDL17). (B) Telomerase expression in primary and immortalized cells. Telomerase activity was analyzed by the telomeric repeat amplication protocol (TRAP) assay. Lane 1, negative control (no cell lysate); lane 2, positive control; lane 3, gingival fibroblasts; lane 4, IGF; lane 5, PDL; lane 6, IPDL. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Assay for telomerase activity

Expression of telomerase activity in IPDL and IGF cells was analyzed by the telomeric repeat amplication protocol (TRAP) assay (Fig. 3B). Telomerase activity was overexpressed in IGF and IPDL cells, compared with their parental cells.

Intracellular levels of cell cycle regulatory proteins

In immortalized gingival fibroblasts and periodontal ligament cells at the 60th passage, with or without osteogenic supplements, the levels of proteins p53 and pRb were lower than in primary cultured cells. In contrast, the levels of proteins p21 and p16 were higher than in primary cultured cells (Fig. 4). The treatment of the IPDL and IGF cells with osteogenic supplements significantly reduced the expression of pRb and p53, and enhanced the expression of p21 and p16. These results suggest that the increase in cell proliferation and the lengthening of the G_2/M phase caused by the E6/E7 oncogene in IGF and IPDL cells are related to p53 and pRb degradation and to the induction of p16 and p21 expression.

ALPase assay

Because ALPase plays a role in mineralization, the effect of osteogenic supplements on ALPase activity in periodontal ligament and gingival fibroblast cells was examined (Fig. 5).

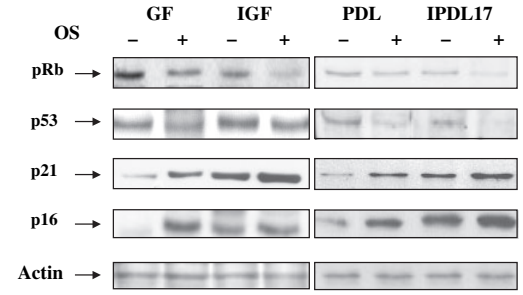

Fig. 4. Western blot analysis for cell cycle regulator (p16, p21, p53 and pRb) protein expression in gingival fibroblasts (GF), immortalized gingival fibroblasts (IGF), periodontal ligament cells (PDL) and immortalized periodontal ligament cells (IPDL17) for 14 d. The protein fractions were extracted, electrophoresed, transferred to membranes and blotted with the respective antibodies. The data shown are representative of three independent experiments. Cells were cultured with or without osteogenic supplement (OS).

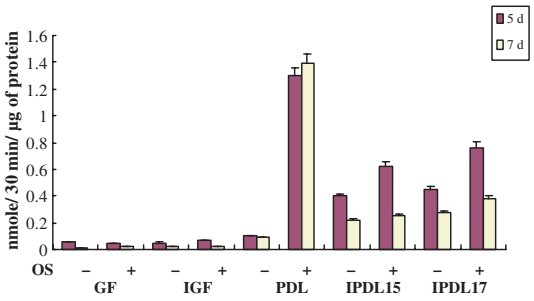

Fig. 5. Alkaline phosphatase activity of primary cultured gingival fibroblasts (GF) and periodontal ligament cells (PDL) at the 5th passage vs. immortalized gingival fibroblasts (IGF), immortalized periodontal ligament cells (IPDL15 and IPDL17) at the 60th passage. The cells were untreated (–) or treated (+) with osteogenic supplements (OS) for 3, 5 and 7 d. OS consisted of 50 μg/ml of ascorbic acid, 10 mM of beta-glycerophosphate, and 10^{-7} M dexamethasone.

During the culture period, osteogenic supplements caused a significant increase of ALPase activity in periodontal ligament cells, but not in gingival fibroblasts or IGF cells. This effect of osteogenic supplements on periodontal ligament cells increased with culture time during the 7-d culture period. As shown in Fig. 5, IPDL15 and IPDL17 cells also showed osteogenic supplement-induced ALPase activity, but this activity was lower than in periodontal ligament cells.

In vitro mineralization assay

Replicate groups of cells were incubated with or without osteogenic supplements for 7, 14, or 21 d. Periodontal ligament cells treated with osteogenic supplements showed strong mineralization activity at 14 and 21 d, and IPDL cells treated with osteogenic supplements showed weak mineralization activity at 14 d and much increased activity at 21 d. No mineralization was detected in gingival fibroblasts and IGF cells, even if treated with osteogenic supplements until 21 d (Fig. 6).

Intracellular levels of osteogenic markers

As periodontal ligament cells are believed to be osteogenic, we characterized our IGF and IPDL cell lines at the 60th passage for osteoblast-specific markers, including OPN, ON, BSP and BMP-2. Differential expressions of one or more of these markers might indicate the differentiation of periodontal ligament and IPDL cells, in contrast to the differentiation of gingival fibroblasts and IGF cells (Fig. 7).

ON is a major phosphorylated extracellular matrix protein in mineralized tissues, whereas OPN, an early marker of bone formation, is believed to be involved in proliferation and migration of osteogenic and cementogenic cell populations (26). OPN expression was readily observed in periodontal ligament and IPDL cells (lane 1), but not in gingival fibroblasts or IGF cells. This OPN expression was slightly increased by treatment with osteogenic supplements. On the other hand, ON expression without treatment with osteogenic supplements was highest in periodontal ligament cells, followed by IPDL cells, gingival fibroblasts and IGF cells (Fig. 7). After treatment with osteogenic supplements , ON expression dramatically increased in gingival fibroblasts and slightly increased in periodontal ligament and IPDL cells, but not in IGF cells.

BSP is a marker of osteoblasts at the middle stage of differentiation, and expression of this gene is undetectable or minimal in the periodontal ligament (27). Consistent with this observation, BSP expression was rarely detected in our periodontal ligament cells, whereas it was intensive in gingival fibroblasts and IGF cells. IPDL cells showed slightly higher expression of BSP than periodontal ligament cells; however, both periodontal ligament and IPDL cells showed increased BSP expression by the treatment of osteogenic supplements.

Previous studies have shown that periodontal ligament cells express BMP-2 and BMP-4 mRNAs, suggesting that these BMPs may regulate periodontal ligament differentiation (28). In this study, BMP-2 expression in the absence of osteogenic supplements was sparse in gingival fibroblasts and IGF, and weak in periodontal ligament and IPDL cells until 14 d of culture, while the BMP-2 expression in the presence of osteogenic supplements markedly increased in periodontal ligament cells and slightly increased in IPDL cells.

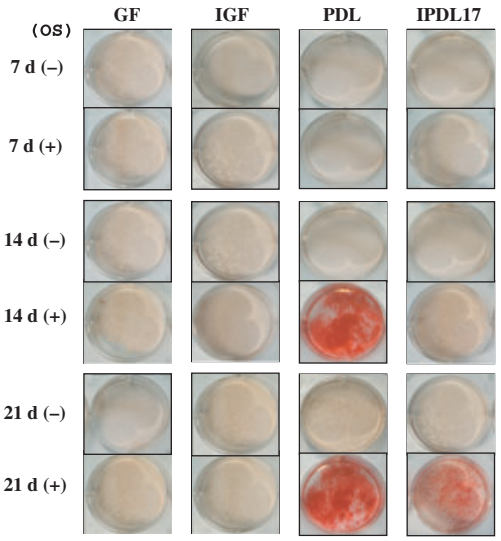

Fig. 6. Formation of calcification nodules in gingival fibroblasts (GF), immortalized gingival fibroblasts (IGF), periodontal ligament cells (PDL) and immortalized periodontal ligament cells (IPDL17) by Alizarin red stain. Cells were cultured with or without osteogenic supplement (OS).

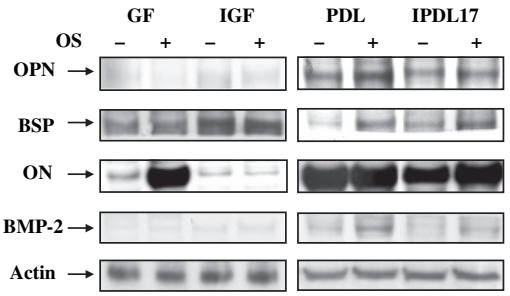

Fig. 7. Western blot analysis for expression of osteoblastic differentiation marker protein, such as osteopontin (OPN), bone sialoprotein (BSP), osteonectin (ON) and bone morphogneic protein-2 (BMP-2), in gingival fibroblasts (GF), immortalized gingival fibroblasts (IGF), periodontal ligament cells (PDL) and immortalized periodontal ligament cells (IPDL17). The cells were cultured with or without osteogenic supplements for 14 d, and then subjected to the procedure described in the legend to *Fig. 6*. The data are representative of three independent experiments. Cells were cultured with or without osteogenic supplement (OS).

Levels of periostin, S-100A4 and PDLs17 mRNA

Periostin, S100A4 and PDLs17 were previously reported to be differentially expressed in periodontal ligament cells (13). Higher levels of mRNA encoding periostin, S100A4 and PDLs17 were expressed in periodontal ligament and IPDL cells at the 60th passage, while these mRNAs were rarely expressed in the gingival fibroblasts and IGF cells (Fig. 8).

Discussion

In the present study, we established and characterized immortalized periodontal ligament and gingival fibroblast cell lines using the HPV16 E6/E7 oncogene, promoting research into the cytodifferentiation mechanism and function of periodontal tissue. This report constitutes the first account of successful immortalization of human gingival fibroblasts and periodontal ligament cells by HPV16 transfection, providing IPDL cells which conspicuously show the periodontal ligament cell-specific markers and osteogenic cytodifferentiation contrast to IGF cells.

Spontaneous immortalization of human cells in primary culture is highly improbable, in contrast to primary cell cultures of rodent origin. This difference arises from the presence of two inherent restraints on the *in vitro* proliferation of human cells (29). These restraints – M1 senescence and M2 crisis – are not present in rodent cells. Therefore, permanent cell lines from various human tissues or organs have instead been propagated from tumors or generated by the infection of primary cell cultures with different oncogenic virus (e.g. Epstein–Barr virus, SV40, HPV16).

The genes encoding HPV16 E6/E7[25] and the SV40 large T antigen are examples of oncogenes used for immortalization of cell lines, which occurs via interaction of their gene products with cell cycle regulatory factors (16,22). The SV40 large T antigen inhibits p53, the cellular gatekeeper for growth and division, by binding and stabilizing it (30). The HPV16 E6 gene product binds to p53 and promotes its degradation; it also inhibits apoptosis and transcriptional activation of p53 after DNA damage (31,32). The HPV16 E7 gene product increases cell proliferation by interacting directly with the retinoblastoma gene product (pRB), which releases the transcription factor, E2F, from Rb complexes, and by inactivating the cyclin-dependent kinase inhibitors $p27^{KIP1}$ and $p21^{WAF-1/Cip1}$ (33,34). Therefore, cells transfected with plasmids containing the HPV16 E6/E7 oncogene under control of a eukaryotic promoter may be able to exit M1 senescence, which normally manifests as a decrease in cell proliferation mediated by p53 and Rb (35).

In our study, we confirmed expression of E6/E7 in the IGF and IPDL cells at the 60th passage (Fig. 3). Although chromosomal integration of the HPV16 E6/E7 sequence could not be shown directly by PCR, stable genomic integration of the coding sequence was inferred from the high passage numbers achieved (36).

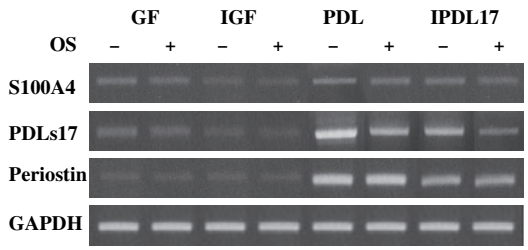

Fig. 8. Expression of periodontal ligament makers, such as S100A4, PDLs17 and periostin, in gingival fibroblasts (GF), immortalized gingival fibroblasts (IGF), periodontal ligament cells (PDL) and immortalized periodontal ligament cells (IPDL17). Cells were cultured with or without osteogenic supplement (OS). RNA samples were isolated and analyzed by reverse transcription–polymerase chain reaction (RT–PCR). Cells were cultured with or without osteogenic supplement. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

The IGF and IPDL cells at the 60th passage grew substantially faster than the primary cultured gingival fibroblasts and periodontal ligament cells (Fig. 2). Analysis by flow cytometry revealed that the numbers of IGF and IPDL cells in G_2/M-phase were markedly increased compared with those of gingival fibroblasts and periodontal ligament cells (Table 1). To elucidate the mechanism by which these changes of IGF and IPDL cells in cell cycle progression are caused, we examined the effect of E6/E7 on cell cycle regulatory proteins. We found that, in IPDL and IGF cells, p53 and pRb were degraded and p21 and p16 levels were increased in comparison with primary cultured cells (Fig. 4). These data indicate that the E6/E7 proteins affect the phosphorylation of pRb and p53, and that their effect on cell cycle progression is relevant to the expression of p16 and p21.

The periodontal ligament contains phenotypically distinct and functional subpopulations of cells from both the fibroblast and osteoblast/cementoblast lineages (5–9). Thus, the periodontal ligament cells have specialized functions that affect the formation and maintenance of the PDL. Periodontal ligament cells have been shown to possess osteoblast-like characteristics, producing ON and osteocalcin and high levels of ALPase, a glycoprotein thought to be involved in the mineralization of calcifying tissues (4–7). Although follicle cells and periodontal ligament cells may have the potential to differentiate into cementoblasts, regulatory factors such as BMP may be required (37); further studies are needed to clarify the cementoblastic differentiation of IPDL.

In our study, periodontal ligament cells exhibited a significant increase in ALPase activity after treatment with osteogenic supplements, and this activity peaked on day 7 (Fig. 5). However, in the IPDL cells that had not been treated with osteogenic supplement, ALP activity was higher than in the periodontal ligament cells. The fact that ALP activity was higher in IPDL cells that had not been treated with osteogenic supplements compared with the periodontal ligament cells, indicates that the response of IPDL cells to osteogenic supplements is not significant and can induce osteogenic differentiation without osteogenic supplements during short-term culture. However, IPDL cells treated with and without osteogenic supplements showed ALPase activity: this activity was decreased at d 7 and lower than the periodontal ligament cells. On the other hand, both gingival fibroblasts and IGF cells exhibited low ALPase activity, even in the presence of osteogenic supplements, until day 7. Therefore, we presume that the gradual loss of ALPase activity in IPDL cells is simply a result of the continuous decrease of cytodifferentiation of osteoblast progenitor cells during the passage of culture.

OPN, BSP and osteocalcin are markers of osteoblasts at the early, middle and late stages of differentiation, respectively (27). Immoralized periodontal ligament cells expressed ALP (15–20), type I collagen (16,18), BSP (15), OPN (15–17,20), ON (16), OC (15–17,19,20), BMP-2 (19) and BMP-4 (19). In the present study, the fact that both periodontal ligament and IPDL cells expressed OPN, BSP, ON and BMP-2 proteins when cultured in the presence of osteogenic supplements, directly indicates the potential of periodontal ligament cells and IPDL cells to differentiate into osteoblasts. This potential was further demonstrated by Alizarin red staining, which showed mineralized matrix upon induction *in vitro* (Fig. 6). In contrast, gingival fibroblasts and IGF cells did not express OPN or BMP-2 when cultured in the presence of osteogenic supplements, although the expression of ON and BSP was observed. These results suggest that gene expression in our HPV16 E6/E7-immortalized human IGF and IPDL cell lines is similar to that in primary gingival fibroblasts and periodontal ligament-derived cells, respectively. Accordingly, Chou *et al.* (26) also reported that gingival fibroblast cells expressed ON, OPN and BSP. The osteoblastic potential of periodontal ligament fibroblasts and gingival fibroblasts could be attributed to the presence of precursor cells important in the repair and regeneration of the periodontium (26).

Ivanoski *et al.* (38). reported that HPV16-immortalized gingival fibroblasts and periodontal ligament cells expressed similar mRNA levels for ALP, BMP-2 and BMP-4 following treatment with PDGF-BB and IGF-1, whereas the expression of OPN, OC and BSP was greater in periodontal ligament cells compared with gingival fibroblast cells. In contrast, we found that gingival fibroblast and IGF cells did not express OPN or BMP-2 when cultured in the presence of osteogenic supplements, but the periodontal ligament and IPDL cells expressed higher levels of OPN, ON and BMP-2 than gingival fibroblasts and IGF cells. Studies in periodontal ligament and gingival fibroblasts suggest that the periodontal ligament possesses multipotential mesenchymal stem cells, which can differentiate into mineralized tissue-forming cells, such as osteoblasts and cementoblasts.

The present study demonstrates that periodontal ligament and IPDL cells

express putatively specific osteoblast proteins, including periostin, S100A4 and PDLs17 (Fig. 8). This periostin expression in our periodontal ligament and IPDL cells is consistent with studies of other human periodontal ligament cell lines immortalized by SV40T and hTERT (18,20). Therefore, this periodontal ligament-specific marker study provides further evidence that periodontal ligament and IPDL cells are closely related to the osteogenic cell lineage.

Collectively, IPDL cells have high ALPase activity and calcified nodule formation, but their gene expression and osteogenic differentiation pattern in culture differs from that of gingival fibroblasts and IGF cells. Therefore, we suggest that gene expression in the HPV16 E6/E7-immortalized human IPDL cells is similar to that in primary PDL-derived cells, and that the IPDL cells can be used in further periodontal research to characterize and elucidate the molecular mechanisms of osteogenic signaling.

Acknowledgements

This work was supported by Wonkwang University in 2006.

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