Effect of humoral factors from hPDLSCs on the biologic activity of hABCs

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This certifies that the dissertation thesis
of Sang-min Lee is approved.

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이 상 민

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ABSTRACT

Effect of humoral factors from hPDLSCs on the biologic activity of hABCs

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Objectives: The human periodontal ligament stem cells (hPDLSCs) and human alveolar bone–derived stromal cells (hABCs) seem to be closely involved in the maintenance of alveolar bone in an anatomically indirect manner; however, there is little study on this matter. Therefore, the effect of hPDLSCs on the osteoclastogenic, osteogenic, and adipogenic differentiation of hABCs was evaluated, focusing on the humoral factors released by hPDLSCs.

Materials and methods: Human periodontal ligament stem cells and hABCs were isolated and characterized. hPDLSCs were indirectly cocultured to observe the in vitro effect of humoral factors released from hPDLSCs on the osteoclastogenic, osteogenic, and adipogenic differentiation of hABCs. Human gingival fibroblasts (hGFs) were utilized as positive control.

Result: Isolated cells demonstrated the presence of stem cells within. Indirect coculture of hPDLSCs greatly inhibited osteoclastogenesis by hABCs. Osteogenesis/ adipogenesis of hABCs was also inhibited by indirect coculture with hPDLSC. The magnitude of regulatory effect from hPDLSCs was significantly greater than that of hGFs.

Conclusion: Humoral factors released from hPDLSCs seemed to modulate the differentiation of hABCs, and the osteoclastogenic, osteogenic, and adipogenic differentiation of hABCs was all inhibited, suggesting the potential role of hPDLSCs in the maintenance of the alveolar bone.

Key Words: stem cells; alveolar bone; periodontal ligament; osteoclast; osteoblasts; adipocytes

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I. Introduction

The presence of the periodontal ligament (PDL) tissue plays an important role in maintaining the homeostasis of alveolar bone, as the absence of PDL tissue following tooth extraction results pronounced alveolar ridge resorption and morphological changes (Tallgren, 1972; Schropp et al, 2003), while the alveolar ridge appears to maintain its volume when a natural tooth or root with viable PDL tissue is preserved within the socket (Salama et al, 2007; Hurzeler et al, 2010).

Previous studies found that while PDL cells appeared to have the dual regulation of osteoclast differentiation (Wu et al, 1999; Kanzaki et al, 2001), little is known about how PDL cells regulate the fate of alveolar bone mass or the exact mechanism underlying alveolar bone resorption after tooth extraction. An understanding of these mechanisms would provide valuable information for clinicians and support the development of more predictable preservation techniques of alveolar bone after tooth extraction.

The PDL tissue functions mainly as a mechanical and structural attachment apparatus between cementum and alveolar bone. However, the cell population in the PDL tissue is highly heterogeneous, and it has been shown that various cell types, including the progenitor cells, are present in the PDL tissue. The presence of mesenchymal stem cells within the PDL tissue has been demonstrated (Seo et al, 2004), and these PDL stem cells (PDLSCs) appear to be responsible for tissue homeostasis, serving as a source of renewable progenitor cells and generating cementoblasts, osteoblasts, and fibroblasts throughout adult life. Other well-known sources of dental-origin mesenchymal stem cells are alveolar bone and its bone marrow. We have demonstrated that these alveolar bone-derived stromal cells (ABCs) presented in vitro characteristics of mesenchymal stem cells and induced new bone formation in an ectopic transplantation model in immunocompromised mice in another report (manuscript in preparation). It was therefore postulated that ABCs also play a major role in the homeostasis of alveolar bone remodeling.

Human periodontal ligament stem cells (hPDLSCs) and human ABCs (hABCs) are located anatomically nearby to each other, and both appear to be fundamentally involved in the maintenance of the periodontal tissues. Currently, it is suspected that hPDLSCs perish with the removal of the associated tooth, which overturns the balance of bone homeostasis into a bone resorption process. Therefore, we hypothesize that hPDLSCs and hABCs communicate with each other somehow to determine the alveolar bone ridge dimensions via the secretion of humoral factors. Therefore, the aim of this study was to determine the effect of hPDLSCs on the

differentiation of hABCs, focusing on the humoral factors released by hPDLSCs. This study used indirect coculture system to imitate the anatomical structure of the periodontium, and differentiation of hABCs was assessed in terms of the three main regulating factors of bone remodeling: osteoclastogenic, osteogenic, and adipogenic potentials.

II. Material and methods

1. Isolation and culture of hPDLSCs, hABCs, and human gingival fibroblasts

Human periodontal ligament stem cells were isolated and cultured according to previously reported protocols (Seo et al, 2004). Briefly, human premolars (n = 12) extracted from three adults with a systemically healthy condition (18–32 years of age) at the Department of Periodontology, Dental College, Yonsei University, for orthodontic treatment were used after receiving the informed consent from the patients. PDL tissues were separated from the root surface with the aid of a scalpel and were minced into the smallest size possible. hABCs were also obtained from the alveolar bone of eight donors during dental implant surgery. An osteotomy was performed using a slight modification of a minimalirrigation technique (Flanagan, 2010). The bone particles released during sequential osteotomy were captured in the drill flutes. The obtained bone chips and minced PDL tissues were digested using a sequential digestion method with 30-min intervals in a-minimum essential medium (α-MEM) (GIBCO, Grand Island, NY, USA) containing 2 mg ml^{-1} collagenase type I (WAKO, Tokyo, Japan) and 1 mg ml^{-1} dispase (GIBCO) at 37℃. Single-cell suspensions were obtained by passing the digested tissues through a strainer with a 70-μmpore size (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA), and the cells ($5 * 10^5$) were seeded on to T75 cell-culture dishes containing α-MEM

supplemented with 15% fetal bovine serum (FBS; GIBCO), 100 lM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (GIBCO) and incubated at 37℃ in a 5% CO^2 atmosphere. Single-cell colonies were observed after 3–7 days, and P0 passages of hPDLSCs and hABCs were cultured. Cells at passages P3–P5 were analyzed. Gingival tissue fragments were separated from three donors during the second implant surgery for implantfixture exposure and processed using a tissue explantation method for the isolation of human gingival fibroblasts (hGFs) (Feng et al, 2010). hGFs were passaged and cultured in growth medium on T75 cell-culture dishes.

The study protocol was approved by the Yonsei Institutional Review Board, and written and informed consents were received from all patients.

2. Characterization of hABCs and hPDLSCs

Colony-forming unit assay was performed as previously reported with slight modification (Friedenstein, 1976; Perkins and Fleischman, 1990; Park et al, 2011). Isolated and ex vivo expanded cells from alveolar bone chips and PDL tissues were plated at a concentration of 1.63 cells cm^{-2} onto the 100-mm culture dishes and incubated at 37℃ in 5% CO^2. Colonies derived from single cells started to form after 3–7 days of culture. After 14 days of culture, the culture dishes were fixed with 4% formaldehyde and then stained with crystal violet (Sigma-Aldrich).

Osteogenic and adipogenic differentiation were performed to confirm the multilineage differentiation potential of mesenchymal stem cells (Pittenger et al, 1999; Park et al, 2011). The culture medium used for the osteogenic differentiation comprised a-MEM with 15% FBS (GIBCO), 2 mM L-glutamine, 100 μM L-ascorbic acid-2-phosphate, 1.8 mM KH_2PO_4, 10 nM dexamethasone (Sigma-Aldrich), 100 Uml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (GIBCO). The culture medium for adipogenic differentiation consisted of α-MEM with 15% FBS, 2 mM L-glutamine, 100 μM L-ascorbic acid-2-phosphate, 0.5 mM isobutyl-methylxanthine, 60 μM indomethacin, 0.5 uM hydrocortisone, 10 μM insulin, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. After 4 weeks (osteogenic differentiation) and 2 weeks (adipogenic differentiation) of induction, the cells were stained with Alizarin red stains and Oil Red O, respectively, for evaluation.

The in vivo regenerative potential of hABCs and hPDLSCs was assessed after transplantation into immunodeficient mice (n = 5). hABCs and hPDLSCs (6.0 * 106) were mixed with 80 mg of hydroxyapatite / tricalcium phosphate (HA / TCP) ceramic powder (Biomatlante, Vigneux, France) and then transplanted into the dorsal surface of 8-week-old immunocompromised mice after incubation for 1 hour, as described previously (Kuznetsov et al, 1997; Song et al, 2011). The transplants were harvested after 8 weeks, fixed with 4% formalin, decalcified with buffered 5% EDTA (pH 7.2- 7.4), and then embedded in paraffin. Sections (5 μm) were deparaffinized and stained with hematoxylin and eosin. Slides were observed under light microscope (BX-50; Olympus Optical Co., Ltd., Tokyo, Japan).

For immunohistochemical analysis of human-specific mitochondria (hMito), deparaffinized sections were immersed in 0.3% hydrogen peroxide to block the endogenous peroxidase activity and then separately incubated with primary antibodies (mouse anti-human monoclonal antibody against hMito (Abcam, Cambridge, UK) diluted in phosphate-buffered saline (PBS) (1:200–1:500). As controls, some sections were treated in the same way with the exception of incubation with the primary antibodies. Sections were subsequently counterstained with H-E. Stained sections were analyzed under a light microscope and photographed.

3. Isolation of human peripheral blood mononuclear cells (PBMCs)

Peripheral blood samples were obtained from three volunteers under approved guidelines set by the Yonsei Institutional Review Board. The samples of blood were diluted, and 30 ml of this diluted blood was layered onto 15 ml of Ficoll–Paque plus (GE healthcare Bio-Sciences, Uppsala, Sweden) and centrifuged for 30 min at 900 g to create different cell layers. The interphase containing human PBMCs (hPBMCs) was collected and resuspended in PBS. hPBMCs were washed at least three times in PBS and finally recovered in a-MEM containing 10% FBS (GIBCO) and 1% antibiotics.

4. Direct coculture of hABCs and hPBMC (hABC ／ hPBMC) or hPDLSC and hPBMC (hPDLSC/hPBMC) for osteoclastogenesis

The optimal osteoclastogenic induction time and culture periods were established in a pilot experiment in which cultures were analyzed weekly for 3 weeks (data now shown). hPDLSCs and hABCs were plated separately at cell densities of $1 * 10^5$ on six-well plates (Falcon; Becton Dickinson Labware). At 70–80% confluency, hPBMCs ($5 * 10^6$) were plated directly on the top of the hPDLSC and hABC cultures each for induction of osteoclastogenesis. Direct coculture of hABCs or hPDLSC with hPBMCs was performed in the presence of α-MEM (GIBCO) containing 10% FBS, 100 Uml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 10^{-8} M dexamethasone (Sigma-Aldrich), and 10^{-7} M 1,25-dihydroxyvitamin D3 (Enzo Life Sciences, Farming dale, NY, USA). After 2 weeks, the formation of multinucleated cells(MNCs) was analyzed.

5. Indirect coculture of hPDLSCs and hGFs over the hABC/hPBMC coculture

We evaluated the effects of hPDLSCs on the osteoclastogenesis of hABCs cocultured with hPBMCs and hGFs were used as a control. hPDLSCs and hGFs were, respectively, plated onto the insert with a pore size of 0.4 μm (six-well format cell-culture insert, Falcon; Becton Dickinson Labware) and then indirectly cocultured on the top of the hABC /hPBMC direct coculture for 2 weeks. The subsequent formation

of osteoclast-like cells was measured. The number of osteoclasts with multiple nuclei (i.e., more than two), identified as tartrate-resistant acid phosphatase (TRAP)-positive cells, was calculated. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) for receptor activator of nuclear factor κ B ligand (RANKL) and osteoprotegerin (OPG) was performed as described later.

6. Indirect coculture of hPDLSCs and hGFs over the hABC culture for osteogenic and adipogenic differentiation

Human periodontal ligament stem cells or hGFs ($4 * 10^4$) were, respectively, seeded onto the six-well plate inserts (Falcon; Becton Dickinson Labware), and hABCs ($1 * 10^5$) were cultured onto six-well plates (Falcon; Becton Dickinson Labware) until they reached a subconfluent stage. Indirect coculture of hPDLSCs or hGFs over the hABC culture using osteogenic or adipogenic differentiation medium mentioned earlier was performed, and the total area of mineralized nodule and lipid vacuole formation were measured using an automated image-analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA) after 4 (osteogenic) and 2 (adipogenic) weeks. RNA from the cultured cells was isolated, and qRT-PCR was conducted to evaluate osteogenesis- and adipogenesisrelated mRNA expression.

7. Total RNA extraction and quantitative reverse transcriptase polymerase chain reaction

Directly cocultured cells below the inserts were harvested for the analysis of osteoclastogenesis-, osteogenesis- and adipogenesis-related gene expression using qRT-PCR, according to the manufacturer's instructions for each product. Briefly, total RNA was isolated from in vitro culture specimens using TRIzol reagent (MRC, Cincinnati, OH, USA). Isolated total RNA was used as a template for the synthesis of cDNAwith CycleScriptRTPreMix (Bioneer, Daejeon, Korea). The subsequent PCR amplification utilized the LightCycler® 480 II real-time PCR (Roche Diagnostics, Penzerg, German), ABI 7500 real-time PCR system and software (Applied Biosystems, Foster City, CA, USA), and specific primers (Table 1). The relative levels of mRNA expression were quantified by comparison with the internal standard (b-actin). Each PCR was performed in triplicate with the same total RNA.

8. Statistical analysis

Each in vitro experiment was performed at least three times. Mean and standard deviation values were calculated. Statistical differences were determined by ANOVA and post hoc t-test for multiple comparisons, and unpaired t-test for comparisons between two independent groups. The level of statistical significance was set at $P < 0.05$.

III. Results

1. Characterization of hABCs and hPDLSCs

First, the ability of hABCs and hPDLSCs to form adherent colonogenic cell clusters of fibroblast-like cells was demonstrated by the formation of colonies observed after 14 days (Figure 1a,b,i,h). Differentiation into the osteoblastic and adipocytic lineages further demonstrated the presence of mesenchymal stem cell population within the isolated cells (Figure 1c,d,k,l). We also evaluated the in vivo tissue regeneration activity of the hABCs and hPDLSCs in an ectopic transplantation model using immunocompromised mice, and all animals healed well after surgical intervention. There were no significant complications, and the animals were sacrificed 8 weeks after transplantation. Significant new bone had formed around the material in the hABCs group, while cementum-like tissue was formed in hPDLSCs group (Figure 1e,m). Newly regenerated tissue showed well-mineralized tissue formation under the polarized microscopic observation (Figure 1f,n). Sharpey's fiberlike insertion with collagenous fiber formation was distinctively observed in hPDLSC group.

To verify the source of the osteogenic / cementogenic potential, immunohistochemical staining for hMito was performed. hMito-positive cells were observed within the newly formed tissue, suggesting that the newly formed bone or

cementum was induced by the transplanted hABCs and hPDLSCs (Figure 1g,o). Negative control sections demonstrated lack of stained cells (Figure 1h,p)

2. Direct coculture of hPBMCs with hABCs or hPDLSCs (hABC／hPBMC or hPDLSC／hPBMC) induced osteoclastogenesis

Direct coculture of hABCs or hPDLSCs with hPBMCs induced significant osteoclastogenesis, as evidenced by TRAP staining at 1, 2, and 3 weeks after coculture (data not shown). Mononuclear TRAP-positive cells were present at week 1, and numerous multinuclei cells (MNCs) were evident after 2 weeks in both the hABC /hPBMC and hPDLSC /hPBMC groups. The osteoclastogenesis was observed continuously throughout the 3-week observation period according to our results.

In this study, we used mouse bone marrow (mBM) to induce osteoclastogenesis, as described previously (de Vries et al, 2006) and compared mBM with hPBMC. However, the efficacy of osteoclastogenesis was lower for mBM cells than for hPBMCs under the same culture conditions (Figure 2a). The formation of TRAP-positive cells was greatly increased in the hPDLSC /hPBMC culture; however, the number of MNCs, especially with more than three nuclei, was significantly higher in the hABC/hPBMC culture (Figure 2b).

3. hPDLSCs indirectly inhibited osteoclastogenesis in the hABC / hPBMC coculture

While it was shown that the degree of osteoclastogenesis in the hPDLSC/hPBMC coculture was lower than in the hABC /hPBMC coculture, it is clearly evident that the hPDLSC /hPBMC direct coculture could induce substantial osteoclastogenesis which is in line with previous study (Kanzaki et al, 2001). Interestingly, previous studies have also produced opposing results, whereby the indirect coculture of hPDLSCs with human osteosarcoma cells downregulated osteoclast formation via soluble factors (Wu et al, 1999). To elucidate the exact role of hPDLSCs, especially on hABCs, which are anatomically separated in the periodontium, we investigated the effect of hPDLSCs on hABCs by observing the osteoclastogenesis in an hABC /hPBMC indirect coculture with hPDLSCs.

After 2 weeks of indirect coculture, the hPDLSC humoral factors exerted an inhibitory effect on hABCinduced osteoclastogenesis (Figure 3a,b). Although substantial osteoclast formation was confirmed by hABCs in direct coculture with hPBMCs, the number of osteoclasts formed in indirect coculture (as identified by TRAP staining) was significantly reduced, and significantly fewer MNCs had more than two nuclei. qRT-PCR revealed that the RANKL/OPG ratio in the hABC/hPBMC coculture was decreased when these cells were cocultured indirectly with hPDLSCs in a separate insert (Figure 3c), and the expressions of related mRNAs

including RANK, carbonic anhydrase II, cathepsin K, ICAM-1, c-fms, LFA-1, M-CSF and calcitonin receptor were significantly downregulated
(Figure 4).

We included hGFs as a positive control, and interestingly, an inhibitory effect of these cells on osteoclastogenesis was also observed. However, the inhibitory effect was more significant in the hPDLSC indirect coculture system, based on the results of TRAP staining and qRT-PCR.

Quantitative real time-PCR using samples obtained from the insert also demonstrated that the expression of OPG mRNA in hPDLSCs was greatly enhanced compared to the hGFs (data not shown), suggesting that an increased expression of OPG by the hPDLSCs is one of the inhibitory mechanisms involved.

4. hPDLSCs indirectly downregulated the osteogenic differentiation of hABCs

After observing the direct and indirect effects of hPDLSCs on osteoclastogenesis by hABCs, the effect of hPDLSCs on the osteogenic differentiation of hABCs was evaluated to determine the relationship between hPDLSCs and hABCs in osteogenic homeostasis (Figure 5). Osteogenic differentiation was induced in hABCs under the indirect coculture with hPDLSCs, and it was found that osteogenic differentiation was significantly down regulated via humoral factors from hPDLSCs, and these effects were confirmed after 3 and 5 days, and 1, 2, and 4 weeks (data not shown). The

degree of osteogenic differentiation was observed by area measurement of mineralized nodules stained with Alizarin red and confirmed by qRT-PCR. Osteogenic markers related to calcification, including osteocalcin, osteonectin, and bone sialoprotein (BSP), were significantly decreased by hPDLSCs. Significantly decreased mineralization was also observed in the hGF group; however, the magnitude of that reduction was lower than for hPDLSCs.

5. hPDLSCs indirectly downregulated the adipogenic differentiation of hABCs

We investigated the effect of hPDLSC humoral factors on the adipogenic differentiation of hABCs (Figure 6). The adipogenic differentiation of hABCs detected by Oil Red O staining was significantly downregulated in hABCs after 2 weeks of indirect coculture with hPDLSCs. qRT-PCR also confirmed that adipogenic markers including lipoprotein lipase (LPL) and peroxisome-proliferator-activated receptor ɤ (PPARɤ)-2 are under the humoral control of hPDLSCs, and this pattern was observed again at 4 weeks (data not shown). Unlike in the osteogenic differentiation tests, hGFs did not inhibit the adipogenic differentiation of hABCs at least in mRNA level although there was relevant reduction in lipid vacuole formation; rather, mRNA expression for LPL was slightly increased compared to that in the hABC-only group. The present results suggest that the inhibitory effect of hPDLSCs on the adipogenic differentiation of hABCs is a unique characteristic.

IV. Discussion

There remain unexplained effects of PDL tissue on the homeostasis of alveolar bone. We investigated the relationship between hPDLSCs and hABCs, as these stem cells are important factors for the maintenance of homeostasis in the periodontium. The coculture system used in the present study represents more in vivo-like conditions and made it easier for us to understand the cellular events and the interactions between mesenchymal stem cells and the surrounding microenvironments. Our study is the first to show that the differentiation of hABCs is modulated by the humoral factors released from hPDLSCs and that osteoclastogenesis, the osteogenic potential, and adipogenic differentiation are all involved, in a well-orchestrated fashion, in the mechanism underlying the maintenance of homeostasis of alveolar bone, and its resorption after tooth extraction.

It is well known that osteoclastogenesis can be activated by RANKL-RANK signaling or direct contact (Kanzaki et al, 2001, 2002; Hasegawa et al, 2002).

The current study demonstrated that TRAP-positive MNCs are significantly induced by the direct coculture of hPBMCs with either hABCs or hPDLSCs. It is well known that mononuclear preosteoclastic hPBMCs differentiate into osteoclasts under the influence of certain differential factors released from the cocultured cells of interest. Although both hABCs and hPDLSCs induced osteoclastogenesis in the present study, the degree of induction was significantly lower in hPDLSCs and the number of TRAP-positive MNCs with more than two nuclei was also significantly

lower among hPDLSCs. It could thus be suggested that the potential for osteoclastogenic induction is greater among hABCs, further suggesting that alveolar bone resorption is mediated, at least in part, by osteoclastogenesis, a mechanism that is further enhanced by hABCs than by hPDLSCs. However, further studies are required to establish the exact roles of hABCs on the upregulated osteoclastogenesis after tooth extraction.

Previous studies have demonstrated that PDL fibroblasts inhibit osteoclastogenesis in indirect contact conditions via soluble factors. PDL cells seem to secrete the factors that inhibit osteoclastic differentiation and function (Wada et al, 2001), and it has been suggested that these factors include OPG/ osteoclastogenesis inhibitory factor, as evidenced by the increased levels of OPG in the culture medium (Kanzaki et al, 2001). More recently, it was reported that when hPBMCs are cocultured indirectly with PDL cells, a higher expression of OPG is induced compared to the monoculture condition (Bloemen et al, 2010). However, this inhibitory effect seems to change with the level of osteoclast maturation. Precursors of osteoclasts are derived from the bone marrow and migrate to the PDL space, where they differentiate into mature osteoclasts. Wada et al (2001) reported that obvious inhibitory action by human PDL (hPDL) fibroblasts on osteoclastogenesis was observed during days 6-9 after culture of mBM cells with conditioned medium (CM) from hPDL fibroblast cultures, while de Vries et al (2006) did not observe any effective osteoclastogenic inhibition during the first 5 days after the application of CM from hPDL fibroblasts onto hPBMCs. Although PDL cells are known to inhibit

osteoclastogenesis in the early stages of osteoclast differentiation via OPG, our results also showed that the inhibitory effect of hPDLSC was effective after 1 week of culture. Therefore, we postulate that at least 6 days are required to fully inhibit osteoclastogenesis via the proper formation of OPG and that this effect continues over a 2-week period. The results obtained from the present analysis model are consistent with the clinical phenomenon in which the removal of PDL tissue and concomitant interruption of OPG release increase the osteoclast activity and subsequent resorption of alveolar bone mediated by osteoclasts. It is necessary to conduct further study to investigate the expression of osteoclastogenesis ／ maturation-related genes to determinethe precise mechanism underlying this inhibitory effect.

Interestingly, we also observed a hGF-induced inhibitory effect on osteoclastogenesis, although these cells were used as positive controls. The anatomy of the soft tissues surrounding the teeth includes PDL and gingival tissue, and the cells from these tissues are in close vicinity to enable the regulation of osteoclastogenesis via secretion of humoral factors. We found that the inhibitory effects were significantly greater for hPDLSCs than for hGFs; we postulate that gingival tissue is less deeply involved in the regulation of osteoclastogenesis related to alveolar bone resorption.

Numerous studies have demonstrated that both direct and indirect coculture systems with adequate cells (Lange et al, 2005; Wang et al, 2006) or with various combinations of growth factors (Krampera et al, 2006) can induce the specifically directed differentiation of mesenchymal stem cells and that microenvironmental

factors appear to be crucial for this specific differentiation. Several studies have suggested that the CM from such culture methods could potentially regulate the gene expression of human mesenchymal stem cells, and it has also been implied that there could still be as yet unidentified or unknown factors within the CM that provide a microenvironment that supports the differentiation of certain stem cells toward tissue-forming cells and ultimately the regeneration of lost tissues. One particular humoral factor released from PDL cells has been shown to affect the differentiation pattern and proliferation of mesenchymal stem cells (Mizuno et al, 2008). Furthermore, the CM of an osteocyte-like cell line, MLO-Y4, was found to significantly enhance the bone formation of human bone marrow stem cells (Heino et al, 2004), and that from apical tooth germ cells has been shown to provide a cementogenic microenvironment and successfully induced the differentiation of PDLSCs along the cementoblastic lineage (Yang et al, 2009). Although several studies have consistently demonstrated the stimulatory effects of CM on stem cells (Fan et al, 2008; Luo et al, 2009), there are also contradictory reports that CM can negatively affect the activity of stem cells. Mizuno et al (2008) demonstrated the upregulation of 35 genes by more than twofold, while 32 genes were downregulated when CM from hPDL cells was cultured with mesenchymal stem cells. Unlike the aforementioned CM from MLO-Y4, that from a human prostatic carcinoma cell line, PC-3, markedly blocked the gene expression of osteogenic mRNA markers and inhibited the osteoblastic cell differentiation of fetal rat calvarial cells (Kido et al, 1997). In line with these studies, CM from hPDL cells was found to inhibit the mineralization of rat bone marrow stromal cells in a dose-

dependent manner (Ogiso et al, 1991). The regulatory effect of humoral factors within the CM seems to be largely dependent on the types of cells and microenvironments used. In our results, nodule formation analysis by Alizarin red staining revealed that the humoral factors from hPDLSC significantly inhibited mineralized nodule formation to a greater extent than the monoculture of hABCs; the expression level of related osteogenic markers exhibited a similar pattern on real-time PCR analysis. The present results have clinical implications in that PDL tissue plays an essential role in suppressing the mineralization by alveolar bone cells within the PDL space and preventing ankylosis. A novel gene, PDL associated protein-1 / asporin, which is involved in this inhibitory regulation mechanism, was recently identified, and it also appears to negatively regulate the mineralization of PDL cells (Yamada et al, 2006, 2007).

While the regulations of osteogenesis and osteoclastogenesis are closely linked to maintain the homeostasis of alveolar bone, the imbalance in osteoclast / osteoblast activity resulting from tooth extraction may cause marked alveolar bone resorption in the clinical situation. Recent studies question the additional mechanism underlying the regulation of these processes (Pei and Tontonoz, 2004). It is now suspected that the reciprocal relationship between adipogenesis and osteogenesis is also related to skeletal homeostasis, especially in terms of PPAR ɤ, which is a key transcription factor implicated in adipogenesis (Akune et al, 2004). Adipose tissue is an anatomically important factor that is closely located to bone tissue, and numerous studies have found that adipogenic differentiation is deeply involved in determining

the bone quantity and quality, along with osteogenic activity, in the normal remodeling process or in the regenerative phase. The most significant histologic finding might be the resorption of bundle bone lining the socket wall immediately after tooth extraction. Interestingly, there is a reduction in bone quality and a rapid loss of dimension after tooth extraction (Araujo and Lindhe, 2005). In the early healing period after tooth extraction, the osteogenic potential is increased, and substantial bone growth is observed into the socket. However, the total dimensions of the extraction socket are markedly decreased, and the newly formed bone tissue is replaced by adipogenic bone marrow tissue in the late healing period. These distinct healing differences between the early and late healing phases likely reflect the observation that the osteogenic potential is temporarily induced, while the adipogenic potential is gradually increased over time. In the present study, hABCs exhibited adipogenic differentiation potential under the appropriate stimulus, and humoral factors released from hPDLSCs seemed to have a negative regulatory effect on the adipogenic differentiation of hABCs. These results partially explain the adipogenic change in the alveolar bone after tooth extraction, with concomitant extinction of hPDLSCs, and emphasize the crucial role of both hPDLSCs and hPDL tissue in the maintenance of alveolar bone quality in terms of bone density.

Collectively, the findings of the present study demonstrate that certain humoral factors released from hPDLSCs indirectly exert an inhibitory effect on the hABC-induced osteoclastogenesis, osteogenesis, and adipogenic differentiation of hABCs, which could possibly elucidate the role of hPDLSCs in the maintenance of the

quantity and quality of alveolar bone, and the alveolar bone remodeling after tooth extraction. On the basis of the results of the present study, the pivotal role of hPDLSCs in the homeostasis of whole alveolar bone could be elucidated, and effective preservation technique of alveolar bone in terms of quantity and quality could be developed.

Table

Table 1. Primer sequences and condition for reverse transcription polymerase chain reaction(RT-PCR)

Gene	Primer sequence		Annealing Temperature (℃)	Genbank no.	Product size(bp)
	Forward(5́-3́)	Reverse(3́-5́)			
OPG	CTGCGCGCT CGTGTTTC	ACAGCTGATGAGA GGTTTGTTCGT	62	NM_002546.3	100
RANK-L	CATCCCATCTG GTTCCCATAA	GCCCAACCCC GATCATG	62	NM_033012.3	60
RANK	CCTGGACCAAC TGTACCTTCCT	ACCGCATCGG ATTTCTCTGT	62	NM_003839.2	67
Carbonic anhydrase Π	TGGACTGGCCG TTCTAGGTATT	TCTTGCCCTTT GTTTTAATGGAA	62	NM_000067.2	100
Cathepsin K	CCATATGTGGGAC AGGAAGAGAGTT	TGCATCAATGG CCACACAGAGA	62	NM_000369.2	150
ICAM-1	TGAGCAATGTGC AAGAAGATAGC	CCCGTTCTGGA GTCCAGTACA	62	NM_000201.2	04
c-fms	CCCTCATGTC CGAGCTGAA	CCCTCATGTC CGAGCTGAA	62	NM_005211.3	126

LFA-1	GAGCTGGTGG GAGAGATCGA	GAGGCGTTGC TGCCATAGA	62	NM_002209.2	106
M-CSF	CCGAGGAGGT GTCGGAGTAC	AATTTGGCACG AGGTCTCCAT	62	NM_000757.4	100
Calcitonin receptor	GCATACCAAGG AGAAGGTCCATAT	ATACTCCAGCC GGTGTGTCAT	62	NM_001164737.1	79
Osteocalcin	CAAAGGTGCAG CCTTTGTGTC	TCACAGTCCG GATTGAGCTCA	60	NM_199173.3	150
Osteonectin	GTGCAGAGG AAACCGAAGAG	TCATTGCTGC ACACCTTCTC	60	NM_003118.2	172
Bone sialoprotein	CTGGCACAGGGT ATACAGGGTTAG	ACTGGTGCCG TTTATGCCTTG	60	NM_004967.3	182
PPARc2	ACAGCAAACCC CTATTCCATGCTGT	TCCCAAAGTTG GTGGGCCAGAA	64	NM_015869.4	159
LPL	TGGACTGGCTG TCACGGGCT	GCCAGCAGC ATGGGCTCCAA	64	NM_000237.2	167
b-actin	CAT GTA CGT TGC TAT CCA GGC	CTC CTT AAT GTC ACG CAC GAT	58	NM_001101.3	249

Figures

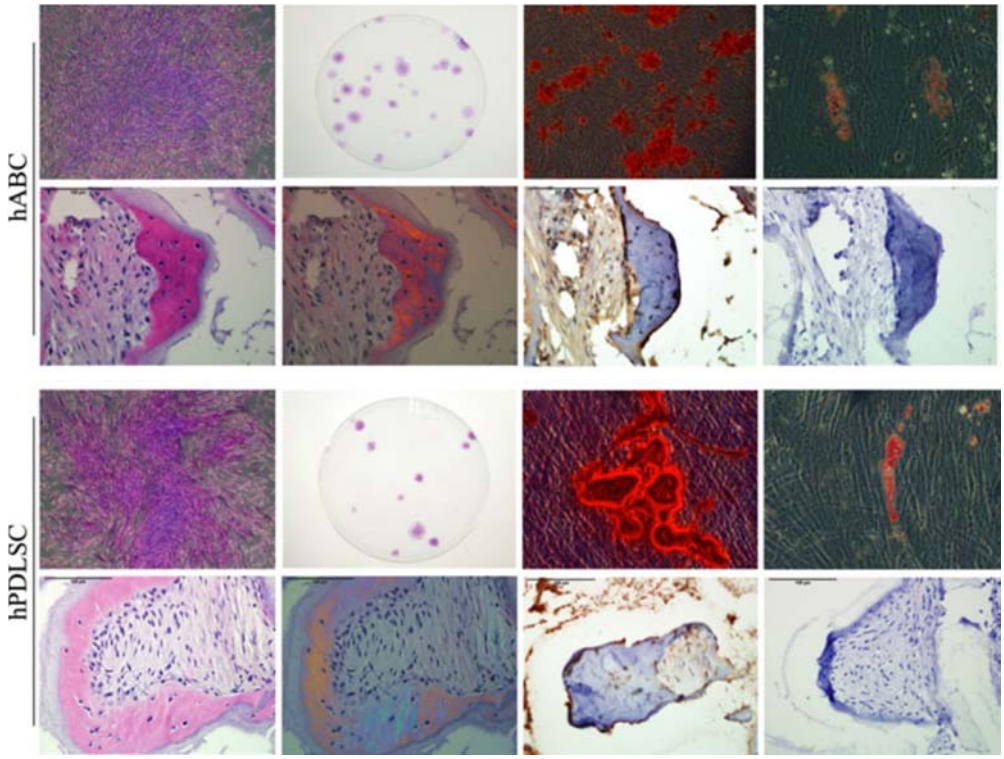

Figure 1. Characterization of human alveolar bone‐derived stromal cells (hABC) and human periodontal ligament stem cells (hPDLSC). (a, b) Colony-forming unit assay demonstrated the colony formation at 14 days after the first seeding (stained with crystal violet, ∗400 and ∗1). (c) Osteogenic differentiation of hABC and hPDLSC groups was successfully demonstrated after 4 weeks of induction (∗400). (d) Adipogenic differentiation was also fully observed after 2 weeks of induction period (∗400). (e) Histologic sections of transplanted materials loaded with hABC and hPDLSC in ectopic transplantation model of immunocompromised mice after 8 weeks (Hematoxylin and eosin, ∗400). (f) Highly mineralized tissue observed under

polarized light microscope (∗400) (g) The immunohistochemical staining against human mitochondria (hMito) demonstrating that the formation of bone or cementum-like tissue is originated from transplanted human cells. hMito-positive cells are observed within the newly formed bone or cementum-like tissue (∗400). (h) Negative control which was stained without primary antibody is shown (∗400). Similarly, isolated hPDLSCs demonstrated colony-forming unit ability, in vitro osteogenic and adipogenic differentiation (i–l). Importantly, highly mineralized cementum and periodontal ligament (PDL)-like tissues were formed (m, n). Immunohistochemical staining against hMito confirmed the cell origin of human donor.

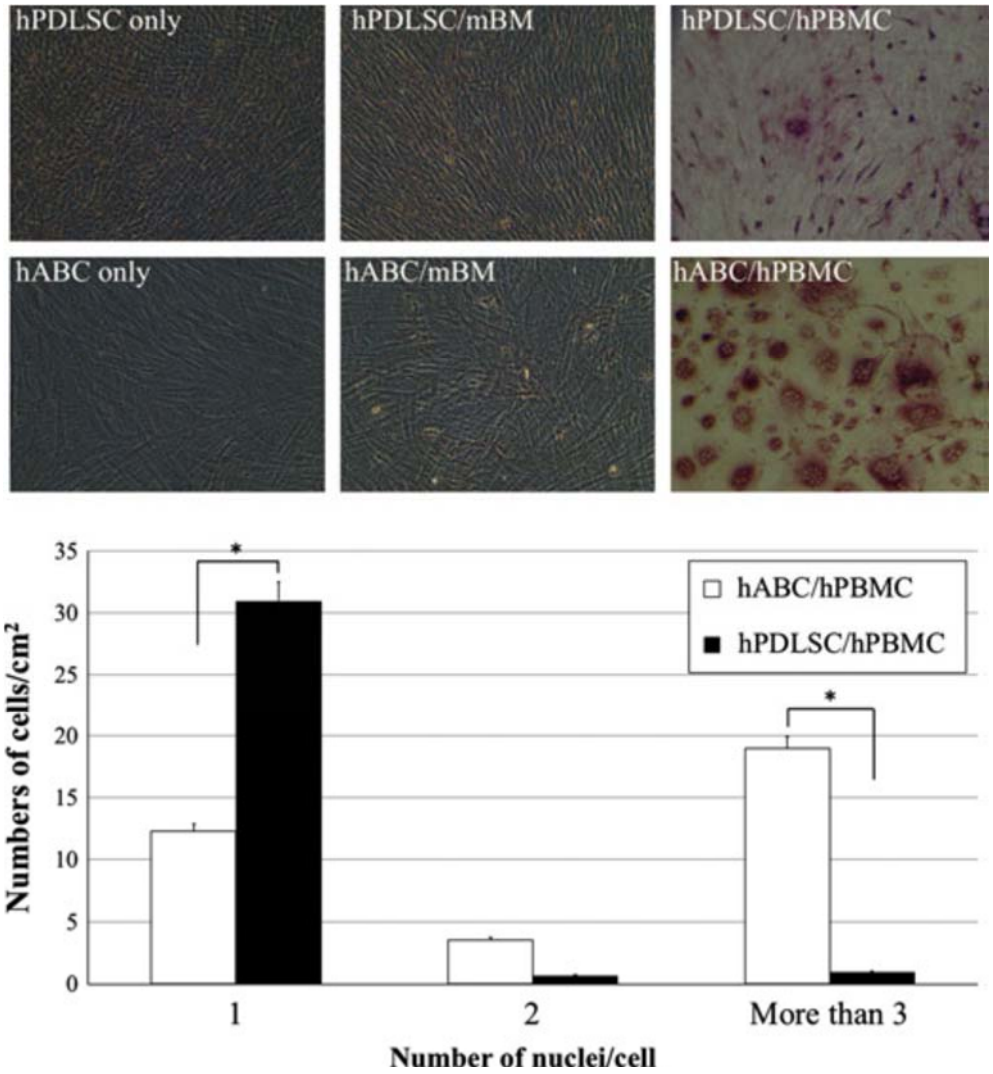

Figure 2. Direct coculture of human alveolar bone‐derived stromal cells (hABC) or human periodontal ligament stem cells (hPDLSC) with human peripheral blood mononuclear cells (hPBMC) induced significant osteoclastogenesis, as evidenced by tartrate-resistant acid phosphatase (TRAP) staining after 2 weeks of induction period. (a) Numerous multinucleated cells were evident after 2 weeks in both the hABC/hPBMC and hPDLSC/hPBMC groups; however, the efficacy of

osteoclastogenesis was lower for mouse bone marrow cells than for hPBMCs under the same culture conditions. Magnification: ∗ 200 (b) Both hABC and hPDLSC induced osteoclast formation; however, the efficacy was statistically higher in hABC group observed by the increased number of cells with multiple nuclei more than three (*P < 0.05)

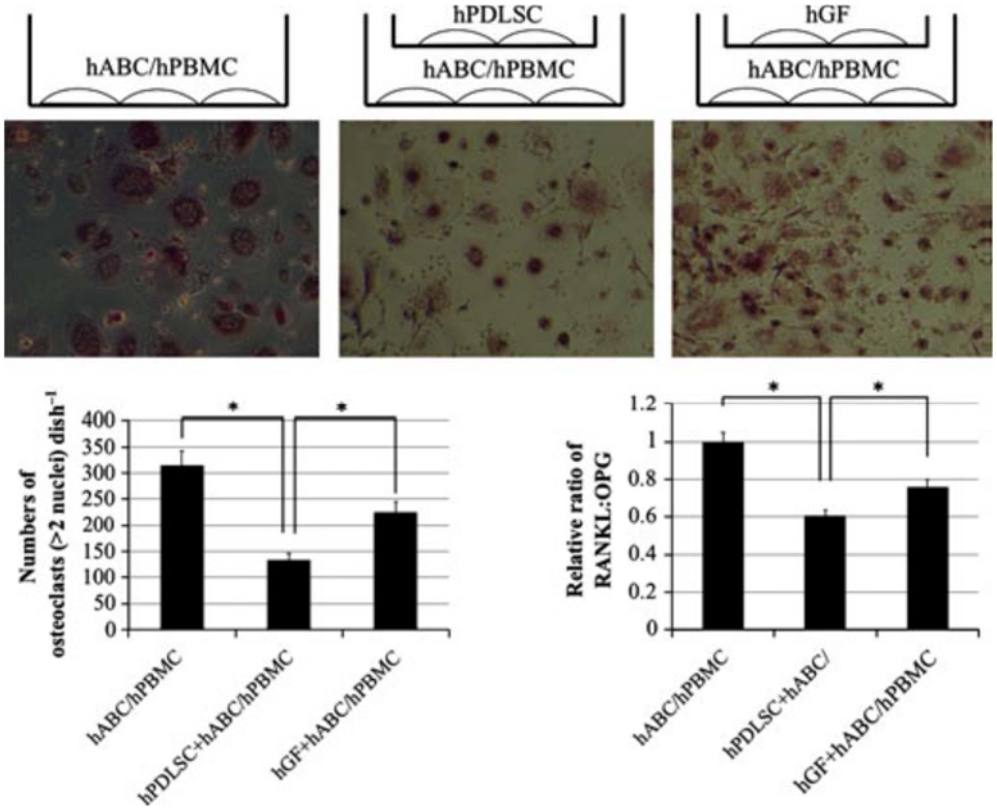

Figure 3. Indirect coculture of human periodontal ligament stem cells (hPDLSC) or human gingival fibroblasts (hGF) with human alveolar bone–derived stromal cells / human peripheral blood mononuclear cells (hABC / hPBCM) direct coculture. (a) Direct coculture of hABC/hPBCM induced significant osteoclast formation; however, the indirect coculture of hPDLSC or hGF has highly downregulated the osteoclastogenesis, especially in hPDLSC group. Magnification: ∗200. (b) The number of osteoclasts formation (>2 nuclei) per dish is illustrated, and the inhibitory efficacy was the greatest in hPDLSC group. (c) Osteoclastogenesis-related mRNA was extracted and compared among groups. The relative ratio of receptor activator of nuclear factor kappa-B ligand (RANKL) over osteoprotegerin (OPG) was similarly decreased in hPDLSC group with statistical significance (*P < 0.05)

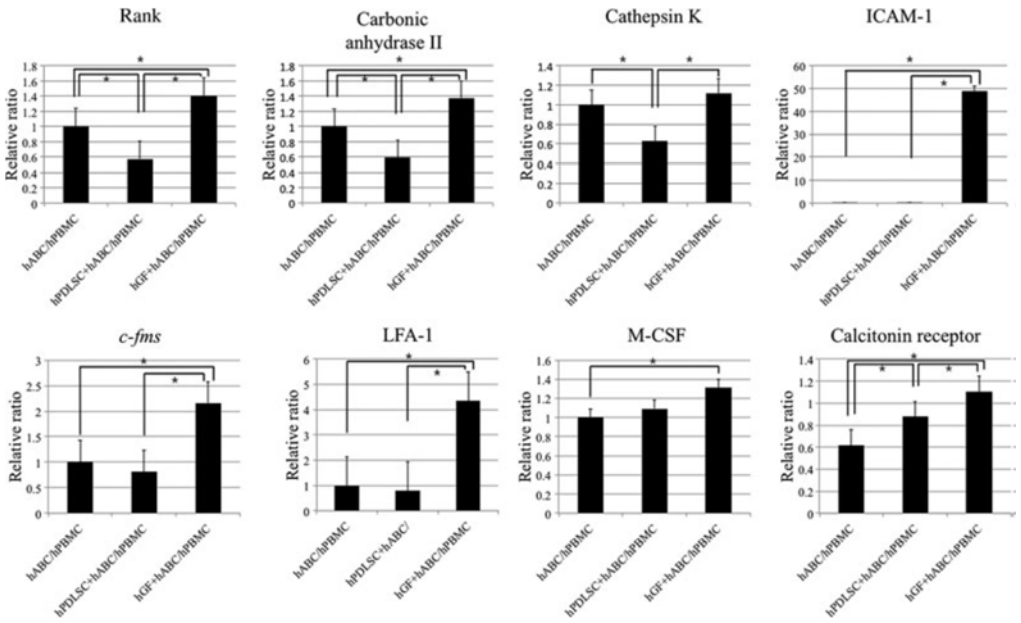

Figure 4. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) results from indirect coculture of human periodontal ligament stem cells (hPDLSC) or human gingival fibroblasts (hGF) with human alveolar bone-derived stromal cells / human peripheral blood mononuclear cells (hABC / hPBMC) direct coculture. Various osteoclastogenesis-related mRNA expressions are illustrated. The experiments were performed at least three times, and the representative data are shown (*P < 0.05).

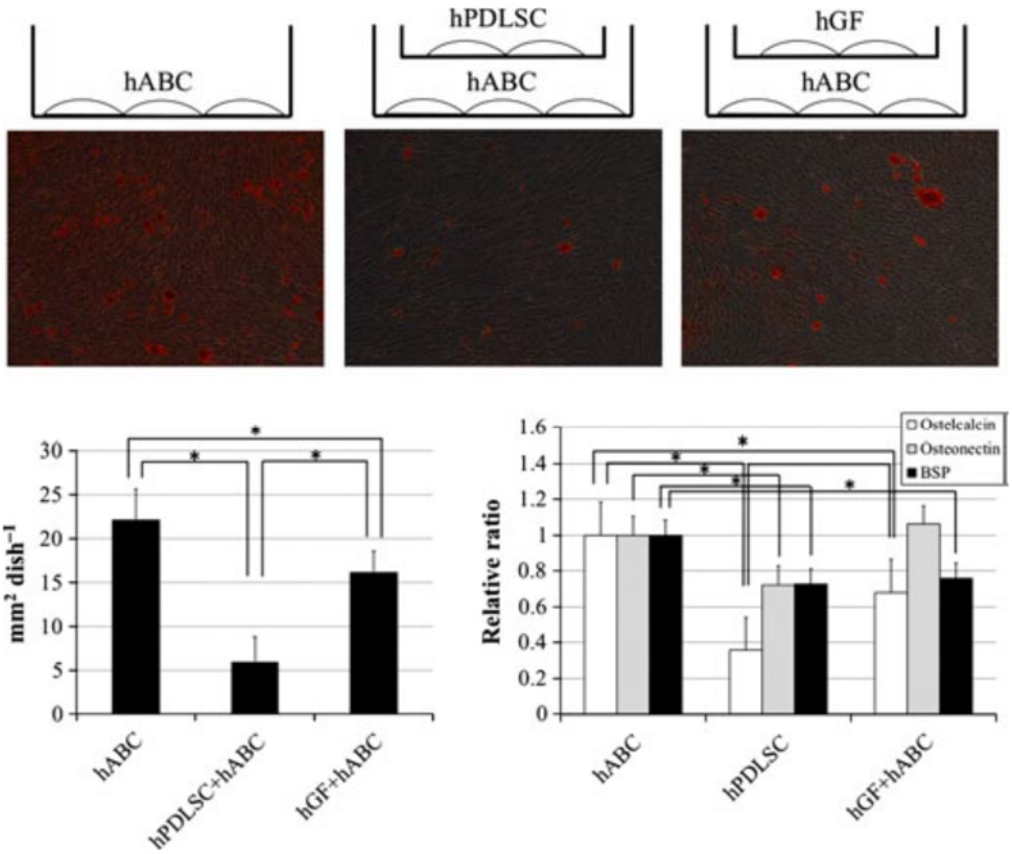

Fig. 5. Osteogenic differentiation of human alveolar bone-derived stromal cells (hABCs) in monoculture or indirect coculture with human periodontal ligament stem cells (hPDLSCs) or human gingival fibroblasts (hGFs). (a) Osteogenic differentiation was induced for 4 weeks and stained by Alizarin red staining. (b) Area of mineralized nodules was measured after osteogenic differentiation, and the nodule formation was significantly decreased under indirect coculture with hPDLSCs. hGFs also showed the reduction; however, the degree was not comparable with hPDLSCs. (c) Osteogenesis-related mRNA expression was evaluated, and hPDLSCs generally exerted inhibitory effects on osteocalcin, osteonectin, and bone sialoprotein (BSP) in comparison with hABCs monoculture or indirect coculture of hGFs. (*$P < 0.05$)

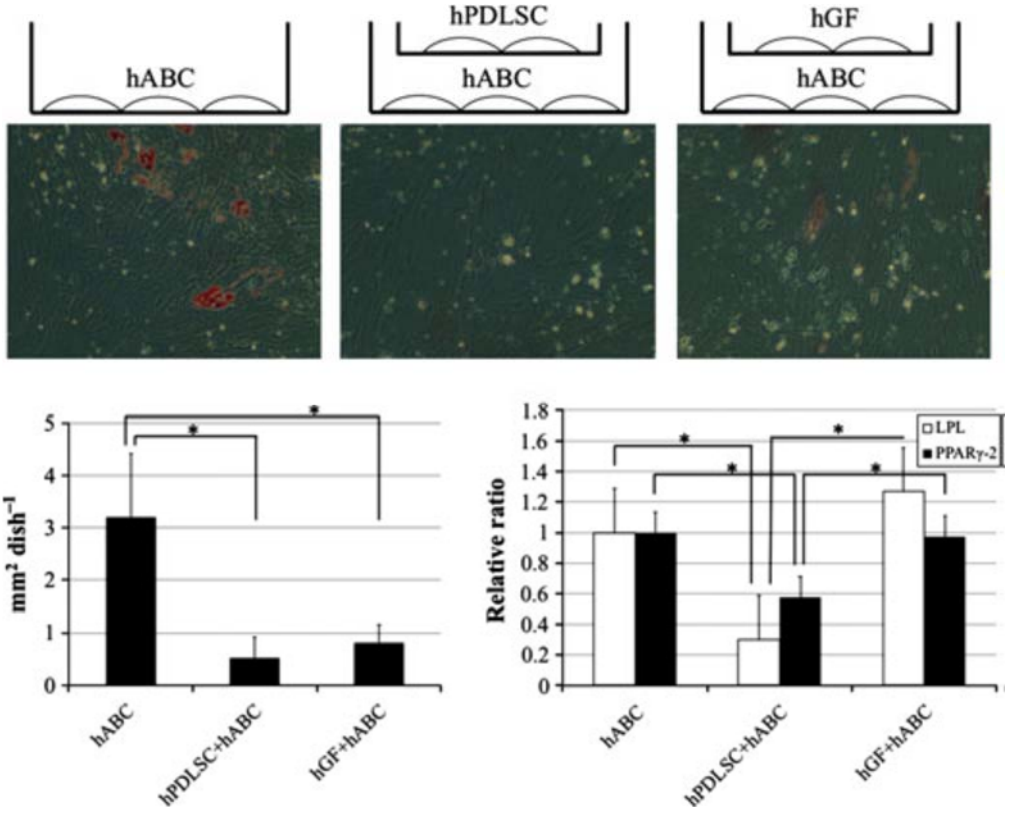

Fig. 6. Adipogenic differentiation of hABCs in monoculture or indirect coculture with human periodontal ligament stem cells (hPDLSCs) or human gingival fibroblasts (hGFs). (a) Adipogenic differentiation was induced for 2 weeks and stained by Oil red O staining. (b) Area of lipid vacuoles was measured after adipogenic differentiation, and the lipid formation was significantly decreased under indirect coculture with hPDLSCs. hGFs also showed the comparable amount of reduction in comparison with hABCs monoculture. (c) Adipogenesis-related mRNA expression was evaluated, and hPDLSCs significantly exerted inhibitory effects on peroxisome-proliferator-activated receptor ɤ 2 (PPARɤ2), and lipoprotein lipase (LPL). However, hGFs did not show any inhibitory effects; rather, it showed the increased expression of LPL (*P < 0.05)

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국문요약

인간 치조골 줄기세포의 생물학적 활동에 대한 인간 치주인대 줄기세포의 체액성 분비 인자의 효과

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이 상 민

목적: 치주인대줄기세포(human Periodontal Ligament Stem Cells: hPDLSC)는 치조골줄기세포 (human Alveolar Bone Stromral Cells:hABC)와 해부학적으로 긴밀히 위치되어 간접적인 방법으로 치조골의 항상성을 유지하는 데 중요한 역할을 하는 것으로 보이지만 이것에 대한 연구가 거의 없다. 따라서 이 연구에서는 hPDLSC 가 hABC 의 파골세포 분화능, 골성 분화능, 그리고 지방성 분화능에 어떤 영향을 미치는지 체액성 분비 인자의 효과에 초점을 맞추어 분석하고자 하였다.

방법: hPDLSC 와 hABC 를 분리하여 세포 특성을 분석하였다. hABC 의 파골세포 분화능, 골성 분화능, 그리고 지방성 분화능에 hPDLSC 로부터

유리되는 체액성 분비 인자가 실험실적 조건에서 어떤 영향을 미치는지 알아보기 위해 hPDLSC 를 간접 배양 기법에의해 배양하였다. 치은섬유아세포(human Gingival Fibroblast: hGF)는 양성 대조군으로 사용되었다.

결과: 분리한 각 세포에는 중간엽 줄기세포의 특성을 보유하고 있음이 증명되었다. hPDLSC 의 간접 배양에의해 hABC 의 파골세포 분화능은 억제되었다. 또한 hABC 의 골성 분화능과 지방성 분화능 역시 억제되었다. hPDLSC 에 의한 이러한 조절작용은 hGF 에 의한 효과보다 컸으며 이는 통계학적으로 유의하였다.

결론: hPDLSC 로부터 유리된 체액성 분비 인자는 hABC 의 분화를 간접적으로 조절한다. hPDLSC 의 간접 배양에의해 hABC 의 파골세포 분화능, 골성 분화능 그리고 지방성 분화능을 모두 억제하는 것으로 보아 hPDLSC 는 치조골의 항상성을 유지하는 데 중요한 역할을 하고 있을 것으로 사료된다.

핵심되는 말: 줄기세포, 치조골, 치주인대, 파골세포, 골아세포, 지방세포