Abstract: The purpose of this study was to evaluate the effects of orthodontic forces (OF) on the proliferation and differentiation of human periodontal ligament stem cells (hPDLSCs). The experimental sample consisted of 6 premolars extracted from 2 patients. After application of OF for 1 month, the hPDLSCs were separated from the primary cultured PDL cells using magnetic-activated cell sorting. The cell proliferation rate was assessed using a 3-[45-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay. The hPDLSCs were cultured in osteogenic medium, and the osteogenic differentiation was analyzed on day 7 and 14 using alkaline phosphatase staining and reverse transcription polymerase chain reaction analyses. The gene expression level of osteogenic markers and angiogenic markers were measured and normalized. The results showed that the application of OF increased the proliferation rates, the expression of osteogenic factors, and the expression of angiogenic factors of hPDLSCs. These findings suggest that OF can serve as a potent positive modulator of proliferation and osteogenic differentiation of hPDLSCs.

Keywords: orthodontic force; human periodontal ligament stem cells; osteogenic differentiation.

Introduction

The periodontal ligament (PDL), a specialized tissue that develops from the dental follicle during odontogenesis, is responsible for creating a tight connection between the cementum surrounding the root of the tooth and the alveolar bone on the surface of the alveolar socket (1). It functions as a site of tissue regeneration, maintains tissue homeostasis by supporting the tooth structure, and provides constant nutrition to the tooth (2).

It has been reported that 5-20% of the adult population in most countries suffer from severe periodontal disease, often resulting in tooth loss that seriously affects the physical and mental health of an individual and their quality of life (3). Recovery of the damaged periodontal tissue plays a key role in periodontal restoration (4).

Recently, human periodontal ligament stem cells (hPDLSCs) have gained popularity for being a possible cell source for tissue regeneration. These stem cells can differentiate, both in vitro and in vivo, into specialized cells such as odontoblasts which, in turn, can generate dentinal structures in vitro (5). Furthermore, hPDLSCs have also been known to regenerate periodontal ligament, cementum, bone, and cartilage in vivo (6). Several
studies have reported that PDLSCs isolated from the PDL tissues of various species demonstrated a capability to differentiate into odontoblasts and induce mineralization in vitro (7,8).

Evidence shows that chemical factors such as cytokines, hormones, and growth factors increase the potency of stem cells to differentiate for tissue regeneration. Moreover, mechanical stimulation may also play an important role in the regeneration of various types of tissues (9,10). Previous studies have reported that the application of mechanical tension on dental mesenchymal stem cells (MSCs) can effectively induce proliferation (9-11). It has also been demonstrated that the ability of hPDLSCs to regenerate and differentiate into various types of cells is enhanced upon application of mechanical stimuli (12), and this has an advantageous effect on hPDLSCs-based periodontal regeneration.

However, the effect of orthodontic forces (OF) on hPDLSCs is still unknown. Therefore, the objective of this study was to evaluate the effect of OF on the proliferation and differentiation of hPDLSC.

Although various studies have previously reported that OF loaded by mechanical stress in vitro can accelerate the osteogenic differentiation of hPDLSCs, there are no studies that have examined the application of this force on experimental teeth in situ (9-11). Therefore, this study applied OF, produced by brackets and nickel-titanium (NiTi) wires, to experimental teeth in situ, which were then extracted and the isolated hPDLSCs were used in the experimental process. This system directly applies the OF to the teeth within the patient’s oral cavity, thus creating a more accurate environment for the examination of the effects of OF on hPDLSCs.

**Materials and Methods**

**Method for mechanical stimulation**
This study included healthy patients (aged 16 to 25 years) who visited the Department of Orthodontics at Pusan National University Dental Hospital. Informed consent was collected from the participants, and the human premolars were extracted after the application of OF (Table 1) using MBT brackets and NiTi orthodontic wires for a period of 1 month. The extraction of the control teeth (left side) and the OF-induced teeth (right side) was executed on the same day. The self-ligating MBT .022 slot brackets were bonded to moderately crowded 1st premolars, and the full-mouth bonding technique was applied using 0.013 inch NiTi wires. The experimental sample consisted of six premolars extracted from 2 patients (Table 1). Procedure was made by split-mouth design for the uniformity in terms of the sizes and the sites of each paired samples (control, OF-induced) within the patients, which will remove a lot of inter-individual variability (13). The use of human tissue for research was approved by the Institutional Review Board (IRB) of the Pusan National University School of Dentistry (PNUDH-2016-032).

**hPDLSCs isolation and culture**
Human periodontal ligament stem cells (hPDLSCs) were isolated and cultured according to previously reported protocols with slight modifications (14). Briefly, the extracted premolars were transferred rapidly from the hospital to the laboratory. The middle third of the roots were carefully scraped with a scalpel and minced to the smallest size possible. The PDL tissue was digested in a solution of 3 mg/mL collagenase type I (Wako Ltd., Osaka, Japan) and 4 mg/mL dispase (Roche Diagnostics, Basel, Switzerland) for 1 h at 37°C. Single-cell suspensions were obtained by passing the cells through a 70-μm cell strainer (Falcon, Heidelberg, Germany). The samples were then centrifuged at 400 g for 4 min and resuspended in an alpha-modification of Eagle’s medium (α-MEM; Welgene Inc., Deagu, Korea) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL). The single-cell suspensions were obtained by passing the cells through a 70-μm cell strainer (Falcon, Heidelberg, Germany). The samples were then centrifuged at 400 g for 4 min and resuspended in an alpha-modification of Eagle’s medium (α-MEM; Welgene Inc., Deagu, Korea) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL). The single-cell suspensions (1 × 106) were then seeded into 100-mm dishes (Nunc, Roskilde, Denmark) and cultured for 1 week in 5% carbon dioxide at 37°C. The single-cell colonies were observed at passage 0 (P0), and the culture medium was changed every 3 days until confluence. The cells from passages P3-P5 were used for the experiments. Limited availability of cells purified from a single tooth made it necessary to utilize pooled-integrated cells for the majority of the experiments, except those shown in Fig. 1.

**Magnetic-activated cell sorting (MACS)**
The hPDLSCs were sorted using magnetic-activated cell sorting (MACS) as per the manufacturer’s protocol (Miltenyi Biotec, Gladbach, Germany). Briefly, the hPDL...
cell suspension ($5 \times 10^6$) was labeled with fluorescein isothiocyanate (FITC)-conjugated antibody (anti-human SSEA-4, 5 μL/10^6 cells, clone MC-813-70, FITC; STEMCELL Technologies, Vancouver, BC, Canada) and anti-FITC Microbeads (5 μL/10^6 cells; Miltenyi Biotec) were used to detect the FITC on the primary antibody. The cell suspension was applied onto the prepared magnetic sorting (MS) column (15). The cells were then sorted into labeled and unlabeled fractions using a MACS column (Miltenyi Biotec).

**Cell proliferation assay**

The cell proliferation rate was assessed using a 3-[4,5-di-methylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, Milan, Italy) assay. The medium used consisted of α-MEM supplemented with 10% FBS, 100 U/mL penicillin (Welgene Inc.), and 100 μg/mL streptomycin (Welgene Inc.), and this was changed every 3 days. The cells were seeded in triplicate in four different 48-well culture plates (Nunc) at a density of $5 \times 10^3$ cells/well. After 0, 24, 48, and 72 hours of incubation, the culture medium in each well was replaced using 300 μL of fresh medium containing 30 μL of MTT solution (0.5 mg/mL MTT in sterile PBS). The plates were then agitated using a shaker for several minutes to extract and solubilize the formazan crystals were dissolved in 300 μL of dimethyl sulfoxide (DMSO; Duchefa Biochemie, Haarlem, Netherlands). The plates were then agitated using a shaker for several minutes to extract and solubilize the formazan. Two 100 μL aliquots from each well were transferred to a 96-well plate (Nunc). After the procedure, the absorbance was read using an Opsys MR microplate reader (DYNEX Technologies Inc., Denkendorf, Germany) with a 570 nm filter. The absorbance data were used to plot the cell growth curves.

**Alkaline phosphatase (ALP) staining**

To evaluate the osteogenic differentiation potential of hPDLSCs, the cells were seeded in a 48-well plate ($5 \times 10^3$ cells/well) and cultured in three different media. To evaluate the osteogenic differentiation potential of hPDLSCs, the cells were seeded in a 48-well plate ($5 \times 10^3$ cells/well) and cultured in three different media.

**Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)**

The mRNA expression of hPDLSCs was evaluated using RT-PCR. Total RNA was extracted from the cell using 1 mL of TRIzol (Life Technologies, Grand Island, NY, USA). Thereafter, 200 μL of chloroform was added and the solution was mixed and incubated for 3 min. After centrifugation at 12,000 g for 15 min at 4°C, the supernatant was discarded. The pellet was washed with 1 mL of 70% ethanol and centrifuged at 4°C for 5 min. The supernatant was discarded, and the pellet was dried and resuspended in 100 μL of DEPC-water at 65°C for 10 min. The nucleic acid concentrations were measured using the NanoDrop ND-1000 spectrophotometer (Technologies Inc., Wilmington, DE, USA). The cDNA was synthesized in a reverse transcription (RT) reaction using 1 μg of total RNA, which was first diluted in 0.5 μg/μL of oligo(dT)18 (Thermo Fisher Scientific, Waltham, MA, USA) and 10 mM dNTPs (Thermo Fisher Scientific). The mixture was heated to 70°C for 10 min in a thermocycler to dissolve secondary RNA structures, and then cooled to 4°C for 2 min. Thereafter, 4μL of 5X RT buffer (Thermo Scientific), 4 μL of 10 mM dNTP (Thermo Fisher Scientific), 0.5 μL of 40 U/μL RiboLock RNase Inhibitor (Thermo Fisher Scientific) and 0.5 μL of 200 U/μL Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific) were added to the mixture and incubated at 42°C for 50 min to allow reverse-transcription. The reaction was inactivated by incubation at 70°C for 15 min. The cDNA was cooled to 4°C and stored at −20°C until further analysis.

The amount of mRNA expression was determined by PCR using AccuPower PCR PreMix (Bioneer, Daejeon, Korea) as per the manufacturer’s protocol. In this way, the gene expression levels of osteogenic markers (ALP, OCN, OPN, RUNX2, and BMP2) and angiogenic markers (VEGF, bFGF2) were measured and normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA levels (Table 2). Primers for PCR were purchased from Bionics (Daejeon, Korea). The amplified cDNA products were separated by electrophoresis on a 2% agarose gel and visualized by UV-induced fluorescence. The obtained band results were quantified by comparing band intensities (relative band intensities...
normalized to respective HPRT band) using ImageJ software (NIH, Bethesda, MD, USA).

**Statistical analysis**

The representative results from the three experiments have been presented as mean ± standard error. Differences between groups were compared using Student’s *t*-test (group comparison), and the results were considered statistically significant at $P < 0.05$ (denoted as *).

**Results**

**Proliferation rate of hPDLSCs**

The effect of OF on the proliferation rates of hPDLSCs was analyzed using an MTT assay, which is a basic method used to test cell viability and proliferation. The assay was performed for each case individually on days 0, 1, 2, and 3 after cell seeding (Fig. 1). Figure 1 shows the effects of OF on the proliferation of hPDLSCs at different time points. Although, there were slight variations between the cases, the overall patterns were largely similar (Fig. 1A), with hPDLSCs obtained from tissues subjected to OF exhibiting enhanced proliferation rates compared to their respective controls after 3 days of culture. The integration of each case also revealed statistically significant increases in cell proliferation in the OF group (Fig. 1B). No significant differences in cell proliferation rates were observed between the two groups on day 1. However, this changed from day 2, with the OF group exhibiting augmented cell proliferation in comparison to the control group. These results suggest that the existence of OF promotes the proliferation of hPDLSCs, which could provide a favorable environment for PDL reorganization.

**ALP activity in hPDLSCs**

The effect of OF on the osteogenic differentiation of hPDLSCs was assessed using *in vitro* osteogenic differentiation culture. The hPDLSCs were cultured in two kinds of OM for 7 days, and the osteogenic differentiation was evaluated using ALP staining (Fig. 2). Upon examination of hPDLSCs cultured for 7 days in the control medium, OM1 (50 μg/mL ascorbic acid, 10 mM β-glycerophosphate), and OM2 (OM1 + 100 nM dexamethasone), the highest ALP activity was observed in the OM2 group and this was selected as the osteogenic differentiation medium for the remaining experiments. In accordance with previous cell proliferation results, the OF group exhibited markedly accelerated osteogenic differentiation (Fig. 2A). Moreover, quantitative analysis of the ALP staining results also confirmed the statistically significant increase in osteogenic differentiation observed in the OF group cultured in OM2 (Fig. 2B). Specifically, the ALP activity significantly increased to 50% in OM1 and 92% in OM2 when compared to the control group ($P < 0.05$). OF significantly increased the ALP activity in OM2 in comparison to the control group ($P < 0.05$). Collectively, these results indicated that osteogenic differentiation was positively regulated in an OF-induced microenvironment. OM2 had the greatest effect on ALP activity and was, therefore, selected as the osteogenic differentiation medium for the remaining experiments.

**Expression of osteogenic markers**

The effect of OF on the osteogenic differentiation of hPDLSCs was assessed by reverse transcription polymerase chain reaction (RT-PCR) analysis. Figure 3

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**Table 2** Primer sequences used for RT-PCR analyses

| Target genes | Sequences | Annealing temperature/°C | Size/bp | Cycles |
|--------------|-----------|--------------------------|---------|--------|
| ALP          | F: 5’-ACGTGGCTAAGAATGTCATC-3’ R: 5’-CTGGTGATCGGATGTCCCTTA-3’ | 55       | 475     | 30     |
| OCN          | F: 5’-CTGGCACAGATCCCTCAAAATA-3’ R: 5’-AGCCATTGATACAGGTTGGG-3’ | 57.5     | 322     | 30     |
| OPN          | F: 5’-CCACACAGCTCCTCAAGAAT-3’ R: 5’-CATAACTGTCTCTCCACCG-3’ | 58       | 467     | 30     |
| RUNX2        | F: 5’-ATTTCAGCTGGTGTTGT-3’ R: 5’-GGCAAGTGAGCCATCGTCAAG-3’ | 58       | 317     | 30     |
| BMP2         | F: 5’-GACCACGTCTCTTCTAGC-3’ R: 5’-GCATCTTGACGTCTTCTCG-3’ | 57       | 468     | 27     |
| VEGF         | F: 5’-GAGATATCAGGCTTCCTCAGGAAACCATGAACACCTTCTGCT-3’ R: 5’-GAGCATGCGCTTCCTGGCCGGGTTTCACCAGC-3’ | 65       | 500     | 30     |
| bFGF2        | F: 5’-ACGTGGCTAAGAATGTCATC-3’ R: 5’-CTGGTAGAGCGATGTCCCTTA-3’ | 57.5     | 416     | 30     |
| HPRT         | F: 5’-ACGTGGCTAAGAATGTCATC-3’ R: 5’-CTGGTAGAGCGATGTCCCTTA-3’ | 58       | 492     | 30     |
shows the mRNA levels of the hPDLSCs after 0, 7, and 14 days of culture. A gradual increase in the osteogenic transcription factors (BMP2 and Runx2) was observed in the OF and control groups when cultured under OM2 conditions (Fig. 3A). On the day 7 and 14, the mRNA levels of osteogenic transcription factors (BMP2 and Runx2) in the OF group showed significantly higher levels than those of the control group (Fig. 3B). This result is of particular importance as increased Runx2 and BMP2 expression in hPDLSCs is directly correlated with osteogenic differentiation (16,17). The OF-mediated promotion of osteogenic differentiation was confirmed by a significant increase in the mRNA levels of the early osteogenic marker (ALP) as well as the late osteogenic markers (OCN and OPN) (Fig. 3A). On day 0, the expression levels of all the osteogenic transcription factors (Runx2 and BMP2) and the osteogenic markers (ALP, OCN and OPN) were higher in the control group (Fig. 3B). However, the osteogenic transcription factors (Runx2 and BMP2) exhibited significant increases in the OF group on days 7 and 14 of culture. Interestingly, the band intensities of the osteogenic markers (ALP, OCN and OPN) were significantly higher only on day 7, and there were no definite trends observed on day 14.
of culture. These results suggest that hPDLSCs highly expressed the osteogenic markers (ALP, OCN and OPN) after 7 days of culture. Taken together, the gene expression analyses results (BMP2, Runx2, ALP, OCN and OPN) showed an enhanced osteogenic differentiation in the OF group (Fig. 3), which could support the positive role of orthodontic force on the osteogenic differentiation of hPDLSCs.

Expression of angiogenic growth factors

The effect of orthodontic force on the expression of the angiogenic factors of hPDLSCs was examined after 0, 7, and 14 days of culture (Fig. 4). When hPDLSCs were cultured in OM2, a substantial difference in the expression of the angiogenic factors [vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2)] was observed between the OF and the control groups (Fig. 4A). The total VEGF (VEGF_{total}) was generally upregulated in the OF group compared with the control group after 14 days of culture. More specifically, the expression of the VEGF splice variants, VEGF_{183} and VEGF_{121}, exhibited remarkable increases in the OF group compared to VEGF_{165} after 14 days of culture. The mRNA levels of VEGF_{total} and FGF2 in the OF group were significantly increased compared to the control group following 7 and 14 days of culture. Moreover, the mRNA levels of FGF2 were also increased compared to the control group on days 7 and 14 of culture.

Discussion

The periodontal ligament, located between the cementum in the middle third of the root and the alveolar bone, functions as a shock absorber to withstand stress loads.
applied to the teeth. It is likely that PDL cells, which are continually stimulated by occlusal, chewing, mastication, and OF, produce local factors that participate in the maintenance and remodeling of the ligament as well as in the metabolism of the surrounding tissues. Moreover, previous studies have reported that the effects of mechanical stimulation on cells are dependent on the intensity, duration, and frequency of the forces (18-20). Several studies have shown that tensile stresses increase cellular activities (21,22), while others have reported that cyclic strains regulate the differentiation of the cells (23). Another study reported that alkaline phosphatase, osteocalcin, and collagen I and III levels increased in bone marrow stromal cells in the presence of 8% strain (24). Wei et al. reported that increased osteogenic differentiation was observed in hPDLSCs exposed to 10% equibiaxial strain at 1.0 Hz for 12 h. Furthermore, increased ALP activity and markedly changed morphology were also seen in the stretched hPDLSCs (10). Simmons et al. investigated the effect of cyclic distortion on the proliferation and differentiation of human MSCs (hMSCs) (24). However, to date, little is known about the precise contribution of OF to the in vitro behaviors of hPDLSCs, including proliferation and osteoblastic differentiation. Therefore, this study sought to determine whether OF induced an increase in proliferation and differentiation of hPDLSCs into osteoblastic/ odontoblastic cells, and ascertain whether the increased osteogenic factors played a role in angiogenic factor production.

To examine the effects of OF on the cell proliferation and differentiation, we used a primary cell culture system of hPDLSCs from the extracted teeth after applying the OF in vivo. Previous studies used artificial static mechanical forces in vitro to imitate the OF in vivo. However, in the present study, we have applied OF directly to the patients’ teeth in their oral cavity so as to create a more realistic OF-induced environment for the hPDLSCs. Moderate OF were applied for a period of one month using MBT brackets and 0.013 inch NiTi ligations. These forces increased the expression of osteogenic transcription factors (BMP2, and RUNX2) and osteogenic markers (ALP, OCN, and OPN) in hPDLSCs. Since BMP and RUNX2 are transcription factors of osteogenesis and are associated with hard tissue formation, the osteogenic marker mRNAs (ALP, OCN and OPN) were seen to subsequent increase and favor the production of mineral deposits that normally occur in response to external forces (25). We hypothesized that exposure to OF regulated the increase in the proliferation and osteogenic differentiation of hPDLSCs and, based on the results, it was proposed that OF stimulated the differentiation of hPDLSCs via the osteogenic pathway. Since hPDLSCs are anatomically located in close proximity to vascular regions, it is not surprising that they have the capacity to produce angiogenic factors that can generate and maintain blood vessels surrounding the teeth (25). Our results are consistent with other reports that the proangiogenic cytokine mRNAs (VEGF and b-FGF) produced by hPDLSCs are increased followed by the increased level of osteogenic mRNAs (BMP2, Runx2, ALP, OCN and OPN).

However, further studies are necessary in order to elucidate the roles of OF in vivo. Although the in vitro studies demonstrated that OF-induced hPDLSCs have greater angiogenic properties, the effect of the vascular potential of hPDLSCs on functional vasculature is still unknown. Tube formation assays will provide a better understanding of the overall net increase in the number of vessels formed in an in vivo model. Furthermore, the limited number of hPDLSC samples in the current study may not fully explain the individual variability observed in the experiments, and further studies that include hPDLSCs from a greater number of patients are necessary. Moreover, the detailed investigation of the cellular responses to the mechanical forces will be needed which takes account for the periods and intensities of the OF.

The results of this study demonstrated that while OF disrupted the microenvironment of the PDL, they also promoted proliferation and differentiation of hPDLSCs through cellular signaling pathways. Therefore, mechanical forces serve as a strong positive mediator for the proliferation and differentiation of hPDLSCs into other types of cells such as osteoblasts. As these cells are usually derived from discarded tissues (i.e., extracted teeth), they may have functional roles to play in therapeutic applications aimed to regenerate tissues and enhance angiogenesis.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant awarded by the Korean government (MSIP) (NRF-2015R1C1A1A01051832 to Yong-Il Kim, NRF-2016R1C1B2012891 to Hyung Joon Kim).

Conflict of interest

The authors declare that they have no conflict of interest with regard to the present study.

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