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Human periodontal ligament cells express osteoblastic phenotypes under intermittent force loading *in vitro*

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1. ABSTRACT

Mechanical strain applied to bone leads to bone remodeling. In the oral cavity, it is unclear how such mechanical force applied to move teeth orthodontically induces alveolar bone remodeling. It is known that osteoclasts are the only cells that are responsible for bone resorption, while the formation and activity of osteoclasts are regulated by osteoblasts. So it is believed that osteoblasts play an important role not only in bone formation but in bone remodeling as well. Therefore, the purpose of this study was to examine the effect of mechanical force on human periodontal ligament (PDL) cells and whether they express osteoblastic characters *in vitro*. *Methods:* Human PDL cells cultured *in vitro* were loaded with intermittently stretching force for 24 hours. The expression of alkaline phosphatase (ALP), osteocalcin (OCN) and osteoprotegerin (OPG) were detected at mRNA and protein levels at 0, 2nd, 4th, 6th, 12th, 24th hours after intermittent force loading. *Results:* Without any stimulation, ALP and OPG mRNA expressions were detected in human PDL cells by in-situ hybridization, but not that of OCN mRNA. ALP mRNA signal was up-regulated and that of OPG was down-regulated by mechanical force within 24 hours. OCN mRNA expression was induced by mechanical force in the late phase of the 24-hours loading cycle. The changes in secreted proteins showed similar results with those seen at the mRNA level. *Conclusion:* Human PDL cells express osteoblastic phenotypes under intermittent force loading and play a role in alveolar bone remodeling.

2. INTRODUCTION

Orthodontic tooth movement occurs during sequential bone remodeling induced by therapeutic mechanical stress (1). It is mediated by bone resorption on the compression side of the periodontal ligament (PDL) and by bone deposition on the tension side of the PDL (2). However, the mechanism by which the signal starts bone remodeling is not yet known. Osteoclasts are the only cells responsible for bone resorption. They derive from hematopoietic cells of the monocyte-macrophage lineage (3, 4). Osteoblasts or stromal cells in bone are essential for osteoclastogenesis (3, 4), making them a key player in studying osteoclastogenesis as well as bone remodeling.

Mechanical stress loaded onto a tooth is transduced to the PDL; then, cells in the PDL respond to the mechanical stress to regulate the resorption and formation of bone matrix by signaling the surrounding cells (5). Generally, "ankylosed teeth", in which the cementum of the root is fused with alveolar bone, cannot be moved by therapeutic mechanical stress because of lack of PDL (6). Therefore, it is suggested that PDL cells are likely to play a pivotal role in bone remodeling during orthodontic tooth movement.

PDL cells are a mixture of mesenchymal cells that have differentiation potential. In earlier experiments, it was found that PDL cells express part of the phenotypic characters of osteoblasts *in vitro* (7, 8). Also, by induction of 1 alpha, 25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2vitD_3$), an osteoclastogenesis stimulator, the osteoblast-like characters in human PDL cells are expressed more readily (9), thus suggesting that such cells have the potential to differentiate into osteoblasts and/or cementoblasts.

Recently, two key factors for osteoclastogenesis,

produced by osteoblasts or stromal cells, have been identified to be important. One is osteoprotegrin (OPG, also called osteoclastogenesis inhibitory factor, OCIF), which inhibits osteoclast formation. The other is the receptor activator nuclear factor kappa B ligand（RANKL）, which supports every step of osteoclastogenesis (10, 11). It is reported that OPG and RANKL are expressed in PDL cells and are regulated by $1\alpha,25(OH)_2vitD_3$ (12, 13, 14), which suggests that PDL cells can be induced to differentiate into osteoblast-like cells and act as a kind of osteoblast-like cells during alveolar bone metabolism.

In this study, we investigated whether *in vitro* mechanical stimulation alone can induce the differentiation of PDL cells to osteoblast-like cells. Alkaline phosphatase (ALP) is closely related with bone mineralization, while osteocalcin (OCN) is regarded as a marker of differentiated osteoblast-like cells. OPG is expressed by osteoblasts, and inhibits osteoclastogenesis. Thus, ALP, OCN and OPG were selected as indices to test the hypothesis that PDL cells play the role of osteoblasts during alveolar bone remodeling induced by orthodontic force.

3. MATERIALS AND METHODS

3.1. Cell culture

Human PDL fibroblasts were cultured *in vitro* (8). The cells were obtained from healthy permanent premolars extracted for orthodontic reasons (n=20, donors' age: 10-13 years old). Informed consent was obtained prior to extraction. The PDL tissues were taken only from the middle of the tooth root to exclude the intermixture of gingiva and dental pulp, and then cultured in Dulbecco's modified eagle medium (DMEM; GIBCO, Grand Island, New York 14072, USA) containing 10% (v/v) fetal bovine serum (FBS; Sanli Ltd., Yazhang Road, Zhejiang, PR China) supplemented with antibiotics (100u/ml penicillin and 100μg/ml streptomycin) at 37°C in a 5% CO_2 incubator. Cells that proliferated from the extracts were passaged four to eight times. Before the following experiments, immunocytochemical staining for keratin was carried out to exclude the intermixture of epithelium, and staining for vimentin to confirm that the cells were originated from mesoderm.

3.2. Application of intermittent mechanical stress

PDL cells were loaded with intermittent mechanical stress using a model *in vitro* (15). Briefly, the silicone membrane attached by a confluent PDL cell layer was stretched in cycle. PDL cells were subjected to 310-320 grams force by a cycle of 3/min for 0, 2, 4, 6, 12 and 24 hours respectively. The cells and the culture medium were harvested at the test time points.

3.3. In-situ hybridization

In-situ hybridization was carried out using the kit (Boshide Bio INC., Wuhan, PR China) as described in the protocol provided by the manufacturer. The odd nucleic acid probes (ALP: 5'- GTAGTCAATT GTAGACTGGT GACGGTCGGG-3'; OCN: 5'- CACAGGTTCG TCCTCCCGTC GCTCCATCAC -3'; OPG (multiphase): [1] 5'-TAGGT AGGTA CCAGG AGGAC ATTTG TCACA-3'; [2] 5'-TGTAT TTCGC TCTGG GGTTC CAGCT TGCAC-3'; [3] 5'-TTGTA GGAAC AGCAA ACCTG AAGAA TGCCT-3') were marked by digoxin. Non-probe controls were compared with each sample. Blue granules (displayed by NBT-BCIP, for ALP and OCN) or brown ones (displayed by DAB, for OPG) in cell plasma showed positive results, while no coloured granules appearance meant negative results. The experiments were repeated for three times.

In order to semi-quantify the expression of OPG mRNA clearly, optical density of the in-situ hybridization staining in plasma was analyzed. For each sample of the PDL cells under force loading for 0hr, 2hrs, 4hrs, 6hrs, 12hrs, 24hrs and the negative control, two slices were chosen respectively. All the slices chosen were stained by the same operator at the same time. For each slice, 120 cells in three different visual fields were imported into the LEICA Q550IW Image-analysis System (made in Germany) by microcamera (200×) to measure the optical density in plasma.

3.4. Assay of secreted protein production

The cells were seeded onto the silicone membranes at 3×10^5 cells/membrane. The test group of human PDL cells was loaded with intermittently stretching force, while the control group was not. Each group contained three samples. The culture medium was harvested at 2nd, 4th, 6th, 12th and 24th hours' culture for the test group and the control group as well. Radioimmunoassay (RIA) measured the amount of secreted OCN, and biochemistry tested that of secreted ALP. The amount of OPG released into the culture medium was determined with mouse monoclonal antibody against human OPG by means of a sandwich enzyme-linked immunoadsorbent assay (ELISA) kit (Cosmo Bio INC., Tokyo, Japan) according to the manufacturer's protocol.

3.5. Statistical analysis

Values were expressed as the mean±standard deviation. We analyzed the data for statistical differences using one-way analysis of variance followed by Bonferroni comparisons and Dunnett T3 comparisons with SPSS 10.0 software. Data with a P-value < 0.05 were considered statistically significant. The results of in-situ hybridization were analyzed semi-quantificationally.

4. RESULTS

4.1. Expression of ALP, OCN and OPG mRNA in PDL cells under intermittently stretching force (Table 1, Figure 1 and Figure 2)

Before mechanical force loading, ALP and OPG mRNA expressions were detected in human PDL cells, while that of OCN mRNA was not. After mechanical force loading, human PDL cells exhibited a stronger signal for ALP mRNA, which showed the ALP mRNA was up-regulated by intermittently stretching force, especially at the late phase of the 24-hours loading cycle. In contrast, OPG mRNA was down-regulated, which decreased sharply at the beginning of mechanical force loading, and leveled off

Table 1. The semi-quantificational analysis of the mRNA expressions of ALP, OCN and OPG in human PDL cells under mechanical force loading

	Non-probe control	Mechanical force loading time (hours)					
		0	2	4	6	12	24
ALP	-	+	+	+	+	++	++
OCN	-	-	-	-	+/-	+	+
OPG	-	++	+	+/-	+	+	+

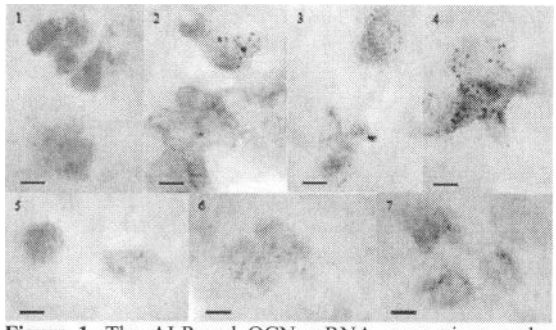

Figure 1. The ALP and OCN mRNA expressions under mechanical force loading tested by in-situ hybridization. Blue granules (displayed by NBT-BCIP) in cell plasma showed positive results, while no coloured granules appearance meant negative results. (1. Non-probe control for ALP and OCN, 1000×; 2. ALP, no force loading, 1000×; 3. ALP, 6 hours force loading, 1000×; 4. ALP, 12 hours force loading, 1000×; 5. OCN, no force loading, 1000×; 6. OCN, 6 hours force loading, 1000×; 7. OCN, 24 hours force loading, 1000×). Scale bars: 4μm. Before mechanical force loading, ALP mRNA expression was detected in human PDL cells, while that of OCN mRNA was not. After mechanical force loading, human PDL cells exhibited a stronger signal for ALP mRNA, which showed the ALP mRNA was up-regulated by intermittently stretching force, especially at the late phase of the 24-hours loading cycle. It exhibited a moderately intense positive signal for OCN mRNA at the late phase of the 24-hours loading cycle, suggesting OCN mRNA was up-regulated in human PDL cells by mechanical force. Experiments were repeated three times with similar results (n=3).

by the end of a 24-hours period. It exhibited a moderately intense positive signal for OCN mRNA at the late phase of the 24-hours loading cycle, suggesting OCN mRNA was up-regulated in human PDL cells by mechanical force. The non-probe control excluded the non-specific stain.

4.2. Secretion of ALP, OCN and OPG proteins by PDL cells under intermittently stretching force (Figure 3)

Without outer stimuli, ALP protein in the culture medium remained at a low level and the secretion amount changed little during 24 hours, while OCN protein even lower. After mechanical force loading, the secretion of ALP protein into culture medium was increased, and exhibited two peaks, one at the beginning of force loading and the other at the late phase of the loading cycle. While in the late of the 24 hours loading period, the expression of OCN in culture medium was increased. On the contrary, the OPG protein secreted was decreased by the induction of mechanical force. The change trends of ALP, OCN and OPG at protein level were in coincidence with those at mRNA level.

5. DISCUSSION

In the present study we have extracted human PDL cells from premolars removed for orthodontic purposes. Therefore it offers an opportunity to study the effect of force application on human PDL cells. The results show that mechanical stress stimulates PDL cells' differentiation into osteoblast-like cells as well as inhibits the expression of OPG, a factor that inhibits osteoclasts formation, thereby enhances bone resorption. Recently, our unpublished data found that mechanical force application led to a significant increase in the expression of RANKL which is essential for osteoclasts formation on day 3 after force application. Thus show that mechanical force leads to bone remodeling through the interaction of regulatory factors such as OPG and RANKL and others expressed by PDL cells. Bone remodeling is bone formation and bone resorption. In the present study, we followed the pattern of expression of alkaline phosphatase (ALP) as an indication of osteogenesis. ALP is an enzyme closely associated with the mineralization process (16). It is the marker of osteoblast phenotype and the marker of osteoblastic differentiation. Clinically, an increase in ALP was detected in gingival crevicular fluid with orthodontic tooth movement (17). In earlier studies, it was found that ALP activity in PDL was higher than that in alveolar bone on the tension side at the early phase of orthodontic tooth movement. However, because of complex internal environment, *in vivo* studies do not provide an ideal model for examining the effects of mechanical force. In the present study, ALP activity in human PDL cells showed significant increase with mechanical stress as shown in figure 1 and figure 3-A. These results point out to the expression of osteoblastic characters by the PDL cells that are known to have a high differentiation potential.

Furthermore, we choose to examine the levels of osteocalcin (OCN) and osteoprotegerin (OPG) as markers for osteoblastic characters as well as osteoclastogenesis and subsequent bone remodeling. OCN is one of the extracellular matrix proteins, which has been implicated to have a role in calcification (18). It is a noncollagenous protein of bone released by osteoblasts, and is regarded as the marker of differentiated osteoblasts. Our data demonstrated that OCN was up-regulated under mechanical force loading as shown in figure 1 and figure 3-B. Thus indicating that in response to mechanical strain produced by applied tension, bone formation was triggered as shown by the significant increase in OCN. Thus far, we demonstrated bone formation by showing a significant increase in both ALP and OCN.

As mentioned earlier, bone remodeling is a combination of bone formation and bone resorption. Therefore, in the present study, we followed the level of expression of osteoprotegerin (OPG). OPG is known as a factor to inhibit osteoclastogenesis. It is a secreted glycoprotein

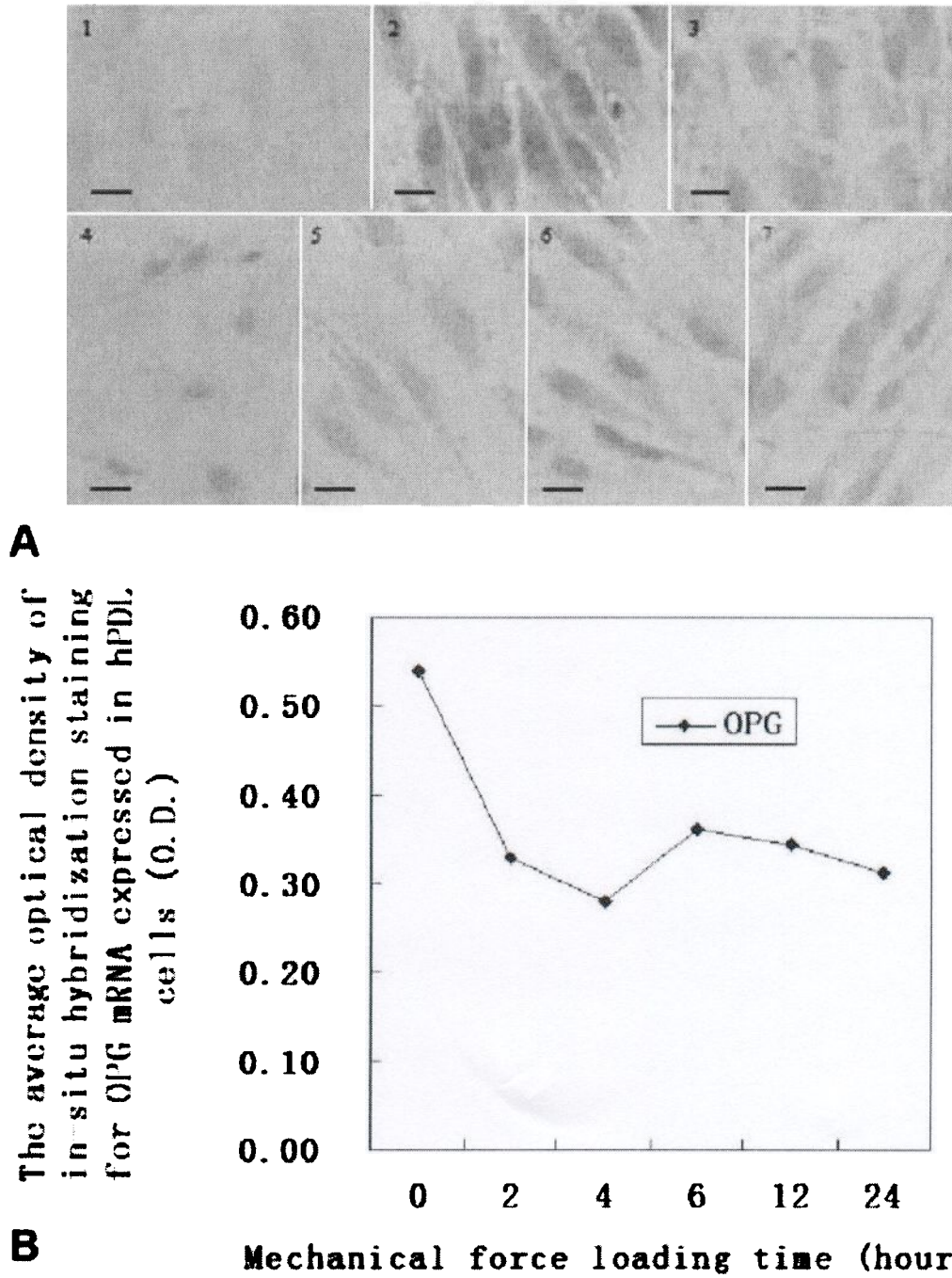

Figure 2. The OPG mRNA expression under mechanical force loading. (A) The results of in-situ hybridization staining. Brown granules (displayed by DAB) in cell plasma showed positive results, while no coloured granules appearance meant negative results. (1. Non-probe control for OPG, 400×; 2. OPG, no force loading, 400×; 3. OPG, 2 hours force loading, 400×; 4. OPG, 4 hours force loading, 400×; 5. OPG, 6 hours force loading, 400×; 6. OPG, 12 hours force loading, 400×; 7. OPG, 24 hours force loading, 400×). Scale bars: 10μm. (B) The optical density of in-situ hybridization for OPG mRNA in plasma of PDL cells under mechanical force loading (n=120). Before mechanical force loading, OPG mRNA expression was detected in human PDL cells. After mechanical force loading, OPG mRNA was down-regulated, which decreased sharply at the beginning of mechanical force loading, and leveled off by the end of a 24-hours period. Experiments were repeated three times with similar results (n=3).

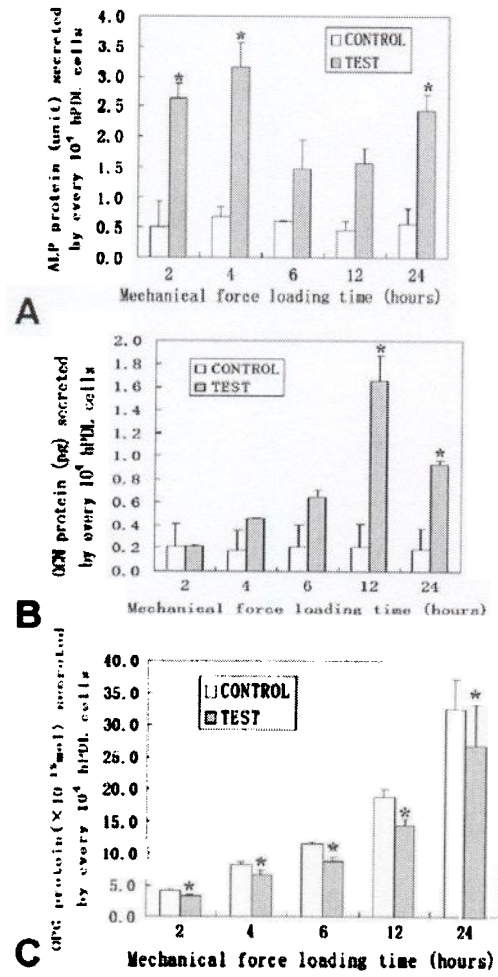

Figure 3. Secreted ALP, OCN and OPG proteins in culture medium of human PDL cells under intermittently stretching force. The test group of human PDL cells was loaded with intermittently stretching force, while the control group was not. Each group contained three samples. The culture medium was harvested at 2nd, 4th, 6th, 12th and 24th hours' culture for the test group and the control group as well. (A) ALP protein in the culture medium. Without outer stimuli, ALP protein in the culture medium remained at a low level and the secretion amount changed little during 24 hours. After mechanical force loading, the secretion of ALP protein into culture medium was increased, and exhibited two peaks, one at the beginning of force loading and the other at the late phase of the 24-hours loading cycle. (B) OCN protein in the culture medium. Without outer stimuli, OCN protein in the culture medium remained at an even lower level and the secretion amount changed little during 24 hours. While in the late of the 24-hours loading period, the expression of OCN in culture medium was increased. (C) OPG protein in the culture medium. The OPG protein secreted was decreased by the induction of mechanical force, which happened from the beginning of force loading. An asterisk (*) represents significant change from the control value ($P<0.05$). Experiments were repeated three times with similar results (n=3).

produced by osteoblasts, and functions as the decoy receptor of RANKL. Opposite to the function of RANKL of supporting osteoclastogenesis, OPG suppresses bone resorption by negatively regulating osteoclast maturation, activity, and survival (10, 11). Results of the current study showed that mechanical strain produced by tension resulted in down-regulation of OPG as shown in figure 2 and figure 3-C. Thus increasing osteoclastogenesis and subsequent bone resorption. These data clearly demonstrated that force application leads to the expression of regulatory factors that are responsible for bone formation and resorption, i.e. bone remodeling.

Although the mechanical force induces bone resorption, it is within physiological limit compared to its pattern of expression when the same cells were exposed to $1\alpha,25\ (OH)_2\ vitD_3$ (14). $1\alpha,25\ (OH)_2\ vitD_3$ is a kind of common bone resorption promoter. In our previous work (14), OPG mRNA expression was found to be down-regulated by $1\alpha,25(OH)_2vitD_3$ in a time dependent manner and decreased continuously up to 6 days of exposure to $1\alpha,25(OH)_2vitD_3$. This indicates that bone resorption continues for a significant period of time demonstrated by the high level of osteoclastic activity. While results of the present study showed that OPG expression leveled off by the end of a 24-hours period. The different pattern of OPG expression when PDL cells were exposed to mechanical force and $1\alpha,25\ (OH)_2\ vitD_3$ suggested the difference of physiological status and pathological status. In physiological conditions, bone formation and bone resorption are balanced under the influence of the factors mentioned above. While in pathological conditions such as periodontitis or periodontal disease, the rate bone resorption is much higher than bone formation.

6. CONCLUSION

Mechanical stress triggers differentiation of PDL cells with high differentiation potential to express osteoblastic characteristics as well as osteoclastic activity. The PDL cells could possibly be a main source of osteoprogenitor cells required for the fastest response required for bone remodeling during orthodontic tooth movement.

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