Cell survival and gene expression under compressive stress in a three-dimensional in vitro human periodontal ligament-like tissue model

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Abstract This study investigated cell survival and gene expression under various compressive stress conditions mimicking orthodontic force by using a newly developed in vitro model of human periodontal ligament-like tissue (HPdLLT). The HPdLLT was developed by three-dimensional culturing of human periodontal ligament fibroblasts in a porous poly-L-lactide matrix with threefold increased culture media permeability due to hydrophilic modification. In vitro HPdLLTs in experimental groups were subjected to 5, 15, 25 and 35 g/cm² compressive stress for 1, 3, 7 or 14 days; controls were cultured over the same periods without compressive stress. Cell morphology and cell apoptosis in the experimental and control groups were investigated using scanning electron microscopy and caspase-3/7 detection. Real-time polymerase chain reaction was performed for

seven osteogenic and osteoclastic genes. Similar extracellular matrix and spindle-shaped cells were observed inside or on the surface of in vitro HPdLLTs, with no relation to compressive stress duration or intensity. Similar caspase-3/7 activity indicating comparable apoptosis levels was observed in all samples. Receptor activator of nuclear factor kappa-B ligand and bone morphogenetic protein 2 genes showed characteristic “double-peak” expression at 15 and 35 g/cm² on day 14, and alkaline phosphatase and periodontal ligament-associated protein 1 expression peaked at 5 g/cm² on day 14; other genes also showed time-dependent and load-dependent expression patterns. The in vitro HPdLLT model system effectively mimicked the reaction and gene expression of the human periodontal ligament in response to orthodontic force. This work provides new information on the effects of compressive stress on human periodontal ligament tissue.

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Introduction

Human periodontal ligament (PDL) tissue is a flexible and well-organized connective tissue that provides the support structure of teeth. PDL connects the tooth cementum to the alveolar bone and also provides support, protection, and the sensation of mastication. It

plays an important role in bone remodeling during the application of orthodontic force, secreting cytokines for osteogenesis on the tension side, leading to bone formation, and, for bone metabolism, leading to bone resorption on the pressure side (Krishnan and Davidovitch 2006; Long et al. 1996; Redlich et al. 2004). Osteoclastogenesis is driven by gene expression induced by mechanical forces and has been investigated in many studies on cultured PDL fibroblasts under mechanical stimulation (Kanzaki et al. 2002; Kim et al. 2013; Wescott et al. 2007).

Cells cultured three-dimensionally in highly porous scaffolds composed of synthetic polymers have attracted high interest in the field of tissue engineering (Langer and Vacanti 1993). This method is also attracting considerable attention for its possible use as in vitro research technique, where it could bridge the gap between traditional two-dimensional (2D) cell cultures and in vivo 3D biological reality (Li et al. 2011). Although many studies have tried to mimic orthodontic force using 2D or 3D cultured periodontal ligament cell models (Kim et al. 2009; Mitsui et al. 2006; Nakajima et al. 2008), the characteristics of these systems including gene expression profiles have shown considerable variability. Previous studies have also not reported cell survival in their models (Sheldrake 1974), raising questions about cell sustainability under compressive stress and whether these in vitro models mimic in vivo conditions. This may explain the observed gene expression variability. The length of experimental observation in previous studies also rarely exceeded 72 h (Kim et al. 2013; Li et al. 2011, 2013; Mitsui et al. 2006; Nakajima et al. 2008), which could compromise cell survival relative to the much longer normal alveolar bone metabolism cycle (Proffit et al. 2006). Further studies on PDL gene expression and a model establishing PDL cell survival in reaction to orthodontic force over longer periods of time are needed.

Recently, we developed an in vitro human periodontal ligament-like tissue (HPdLLT) model by 3D culture of human periodontal ligament fibroblasts in a porous poly-L-lactide matrix. This matrix was hydrophilically modified using ammonia solution, enabling threefold greater permeability of culture media (Liao et al. 2013). Higher culture media permeability directly influences cell living conditions inside the matrix, allowing greater attachment of cells to the 3D matrix in the early stages of cell growth and more accessibility to nutrients and oxygen inside the matrix. As a result of this increased permeability, reliable

cell and extracellular matrix growth inside the 3D matrix was achieved and key PDL genes such as periodontal ligament-associated protein 1 (PLAP-1), alkaline phosphatase (ALP), fibroblast growth factor-2 (FGF-2), and type I collagen (COL1) were upregulated. This suggests that culturing HPdLFs in a hydrophilically modified PLLA matrix is an appropriate way to develop the in vitro PDL model system.

In the current study, cell morphology and apoptosis were evaluated to ensure that this system accurately mimicked the PDL under long-term compressive stress in vitro. The purpose of this investigation was to evaluate osteogenic and osteoclastic gene expression patterns of HPdLLT under various conditions of compressive stress intensity and duration designed to mimic orthodontic force.

Materials and methods

Preparation of in vitro three-dimensional human periodontal ligament like tissue (HPdLLT)

The in vitro HPdLLT was prepared according a protocol published previously (Liao et al. 2013). Briefly, PLLA pellets (average MW = 240,000; BMG, Kyoto, Japan) were dissolved in dichloromethane (DCM; 5 % wt/v; Nacalai Tesque, Kyoto, Japan) and cast with 75–150 µm sized sodium chloride granules (NaCl; Nacalai Tesque) as a porogen. After molding, ammonia (0.05 %; Nacalai Tesque) was applied to hydrophilically modify the porous PLLA matrix (Fig. 1a, b). The thickness and porosity of the PLLA matrix were 400 ± 50 µm and 83.3 %, respectively. Then, 1×10^5 human periodontal ligament fibroblasts (HPdLFs; CLCC-7049; Lonza, Basel, Switzerland) were seeded and centrifuged into a 0.6-cm diameter round-shaped PLLA matrix. The culture medium used in all cell cultures was 10 % fetal bovine serum in Dulbecco's Modified Eagle Medium, along with antibiotic/antimycotic (Nacalai Tesque). Cells were cultured at 37 °C in a 5 % CO₂ incubator. The culture medium was exchanged three times per week throughout the study. HPdLFs were cultured inside the PLLA matrix for 14 days to prepare the in vitro HPdLLT (Fig. 1c).

Application of compressive stress

A classic method was used to apply compressive stress in vitro to the HPdLLTs (Kim et al. 2013; Li et al.

Fig. 1 Microscope image (a) of the poly-L-lactide (PLLA) matrix. Scanning electron microscopic image of PLLA matrix without seeded cells (b) and with cells grown for 14 days (c)

2011; Nakajima et al. 2008). HPdLLTs were continuously compressed using the uniform compression method shown in Fig. 2. In brief, each HPdLLT was cultured in vitro in one well of a 24-well plate (BD Falcon, Franklin Lakes, NJ, USA). A 15-mm-diameter round micro cover glass (Matsunami, Kishiwada,

Japan) was placed over each in vitro HPdLLT. Metal weights were placed on the cover glass to apply compressive stress to the in vitro HPdLLTs. In the experimental group, in vitro HPdLLTs were subjected to 5, 15, 25 or 35 g/cm² of compressive stress for 1, 3, 7 or 14 days, respectively. The control group (0 g/cm²) was cultured for 1, 3, 7 or 14 days and not subjected to compressive stress.

Scanning electron microscopy (SEM)

Samples were rinsed with PBS, fixed in 2 % glutaraldehyde (Nacalai Tesque), post-fixed in 1 % osmium tetroxide (Nacalai Tesque), and dehydrated in a series of increasing concentrations of ethanol in distilled water and finally in *tert*-butyl alcohol (Nacalai Tesque). After *tert*-butyl alcohol was sublimed using a *tert*-butyl alcohol freeze dryer (VFD-21S; Vacuum Device, Mito, Japan), samples were sputter-coated with platinum using an ion sputter (E-1030; Hitachi, Tokyo, Japan). Surface (100×), and cross-sectional images (250×; 1,000×) of all samples were obtained using a scanning electron microscope (S-4000; NEC, Tokyo, Japan) at 3 kV.

Caspase-3/7 detection

Caspase-3/7 activity was observed using a fluorescent staining kit (CellEvent™ Caspase-3/7 Detection Kit; Life Technologies, Tokyo, Japan). All procedures were performed according to the manufacturer's recommended protocol. Briefly, samples were first incubated with reagent in a 5 % CO₂ incubator at 37 °C for 30 min and subsequently observed using an all-in-one fluorescence microscope (BZ-9000; Keyence, Tokyo, Japan). All images were processed using the manufacturer's special analysis software (BZ-II; Keyence).

RNA analysis

Total RNA was isolated from in vitro HPdLLTs using an automated RNA isolation and purification system (QIAcube & RNeasy® Mini QIAcube Kit; Qiagen, Hilden, Germany). Single-stranded complementary DNA was synthesized from mRNA using a cDNA synthesis kit (Transcriptor Universal cDNA Master; Roche Diagnostics, Mannheim, Germany). Real-time polymerase chain reaction (RT-PCR) was performed using a probe kit (Universal ProbeLibrary Set, Human, Roche Diagnostics), master mix (FastStart Universal

Fig. 2 Method used to apply compression stress to mimic orthodontic force on the PDL. Pre-cultured HPdLLTs were compressed continuously in vitro using metal weights loaded on

a piece of thin cover glass placed over the HPdLLTs in each well of a 24-well plate. The metal weight mimicked the orthodontic force and the in vitro HPdLLTs mimicked the PDL tissue

Table 1 Primer sequences used in this study (Sigma-Aldrich, Hokkaido, Japan)

Name	Genebank no.	Probe No.	F/B	Sequences
RANKL	NM_003701	#17	Forward	5'-TGATTCATGTAGGAGAATTAACAGG-3'
			Backward	5'-GATGTGCTGTGATCCAACGA-3'
BMP-2	NM_001200	#49	Forward	5'-CGGACTGCGGTCTCCTAA-3'
			Backward	5'-GGAAGCAGCAACGCTAGAAG-3'
PLAP-1	AY029191	#72	Forward	5'-GGTGGATAACTTCTACTTTTAGGAGGA-3'
			Backward	5'-AAGAAGGGTTTGGCAGAGC-3'
ALP	AB012643	#31	Forward	5'-AGAACCCCAAAGGCTTCTTC-3'
			Backward	5'-CTTGGCTTTTCCTTCATGGT-3'
OPG	NM_002546	#17	Forward	5'-GAAGGGCGCTACCTTGAGAT-3'
			Backward	5'-GCAAACTGTATTTGCTCTGG-3'
COL1	NM_000088	#67	Forward	5'-GGGATTCCCTGGACCTAAAG-3'
			Backward	5'-GGAACACCTCGCTCTCCA-3'
FGF-2	NM_002006	#7	Forward	5'-TTCTTCCTGCGCATCCAC-3'
			Backward	5'-TGCTTGAAGTTGTAGCTTGATGT-3'
GAPDH (control)	NM_002046	#60	Forward	5'-AGCCACATCGCTCAGACAC-3'
			Backward	5'-GCCCAATACGACCAATCC-3'

Probe Master; Roche Diagnostics), and custom-made primers (Sigma-Aldrich, Hokkaido, Japan) using the two-stage program parameters on a PCR system (StepOnePlus™; Applied Biosystems, Foster City, CA, USA). The sequences of order-made primers used in PCR are listed in Table 1. All RNA isolation, cDNA synthesis, and RT-PCR steps were performed according to the manufacturer's recommended protocols.

Statistical analysis

For all experiments, values are reported as the mean $\pm$ SD (standard deviation) for triplicate cell

cultures. Differences were evaluated by analysis of variance (ANOVA) with Tukey–Kramer post hoc tests by using statistical software (Statcel2; OMS Publisher, Tokorozawa, Japan). A value of $p < 0.05$ was considered to be significant.

Results

Morphology of HPdLLT under compressive stress

Scanning electron microscopy images of the in vitro HPdLLT samples under constant compressive stress

Fig. 3 Surface SEM observation of in vitro HPdLLTs ($\times 100$) after application of various compressive stress conditions. In all figures, spindle-shaped cells (arrow A) and extracellular matrix

(arrow B) can be observed on the surface of each in vitro HPdLLT, with no relationship to compressive load duration or compressive stress intensity

or controls were obtained on days 1, 3, 7 and 14. Figure 3 shows the SEM images of the surfaces of the controls (without compressive stress) and in vitro HPdLLT following the application of compressive stress. In some in vitro HPdLLTs (15 g/cm^2 on day 14; arrow A), spindle shaped cells grew densely, covering the PLLA fibers on the surface. The cells connected clearly with each other, and an extracellular matrix was also observed. In other in vitro HPdLLTs (0 g/cm^2 on day 3; arrow B), the extracellular matrix obscured the HPdLLFs. In each case, in vitro HPdLLTs were considered viable and mature. Cross-sectional images (Fig. 4; a local amplification of Fig. 5) revealed similar spindle-shaped cells and the extracellular matrix inside each in vitro HPdLLT, with no relation to the duration of compressive stress or compressive stress intensity.

Caspase-3/7 detection

Caspase-3/7 activity monitors cell apoptosis, and images of the caspase-3/7 activity of in vitro HPdLLTs

subjected to compressive stress and controls were obtained on days 1, 3, 7 and 14. Figure 6 shows green fluorescent apoptotic cells inside in vitro HPdLLTs. Similar densities of green fluorescent cells were observed inside in vitro HPdLLTs with no relation to compressive load duration or intensity.

Gene expression of bone differentiation markers

Evaluated genes were divided into groups based on similar expression patterns over the time course of compressive stress (Fig. 7). The first group included the receptor activator of nuclear factor kappa-B ligand (RANKL) and bone morphogenetic protein 2 (BMP-2). Although their expression showed some significant variation between days 1 and 3, the most significant differences in gene expression were observed on day 14 compared to previous durations at compressive stress intensities of 15, 25 and 35 g/cm^2 . Relative gene expression patterns observed on day 14 at 15 g/cm^2 and 35 g/cm^2 , named the “double peak” because of a characteristic reduction at 25 g/cm^2 , were

Fig. 4 Cross-sectional SEM observation of HPdLLTs ($\times 1,000$) after in vitro application of various compressive stress conditions. In all figures, similar filamentous extracellular matrix and spindle-shaped cells can be observed inside each HPdLLT with no relationship to compressive load duration or

compressive stress intensity. This figure is a local magnification of Fig. 5 ($\times 250$), which shows the extracellular matrix and spindle-shaped cells observed from the surface to the rear side of each in vitro HPdLLT

significantly higher than those observed for other compressive stress durations and intensities. BMP-2 expression from day 1 to day 7 was highest on day 7 at 35 g/cm^2 , and RANKL expression on day 7 also showed the characteristic double peak with significantly higher expression at 35 g/cm^2 compared to day 1 or day 3 at the same compressive stress. The second group included alkaline phosphatase (ALP) and periodontal ligament-associated protein 1 (PLAP-1), both of which showed significantly higher gene expression on day 14 at 5 g/cm^2 compared to other conditions. The third group included basic fibroblast growth factor (FGF-2) and osteoprotegerin (OPG), which showed similar expression regardless of compressive stress intensity, but the day 14 expression levels were stable and significantly higher than those for the previous incubation time periods. Gene expression of FGF-2 on day 7 was significantly higher than that on day 3 at all stress levels, but OPG expression on the same day was not significantly increased. Type I collagen (COL1)

showed relatively stable gene expression regardless of the intensity of compressive stress. There were no significant differences in the expression of COL1 between day 7 and day 14, except under 0 g/cm^2 compressive stress. COL1 expression on days 7 and 14 was significantly higher than that on days 1 and 3 at all compressive stress levels.

Discussion

PDL tissues secrete osteogenic and osteoclastic cytokines in response to mechanical stress for regulation of alveolar bone remodeling around the teeth and to induce orthodontic tooth movement. Morphological observations of cells and the extracellular matrix, in addition to apoptosis evaluated by caspase-3/7 expression, indicated no differences in cell living conditions between various compressive force intensities and durations.

Fig. 5 Cross-sectional SEM images of HPdLLTs ($\times 250$) following the application of various compressive stress conditions. These images reveal the extracellular matrix and spindle-shaped cells observed from the surface to the rear side of each *in vitro* HPdLLT

The clinical value of the optimum orthodontic force was previously reported to be 10–120 g (Proffit et al. 2006), or not greater than the pressure in the blood capillaries, which is approximately 20–26 g/cm² (Schwarz 1932). In studies using 2D cultured cells, however, Mitsui et al. (2006) and Nakajima et al. (2008) reported well-functioning light and heavy orthodontic forces of only 0.5 and 4 g/cm², respectively, although their loading duration was no longer than 24 h. The reported optimum orthodontic force varies considerably between 2D models and clinical experience. In addition, clinical orthodontic visit frequency is usually once every 4 weeks, and tooth movement normally occurs in the first 10 days (Proffit et al. 2006). Data acquired over 24 h are unlikely to mimic the time-dependent gene expression patterns that occur over a 10 day period; therefore it is likely that 2D monolayer culture systems do not have sufficient complexity to mimic *in vivo* reactions of PDL to orthodontic force.

3D cultured tissue models provide additional complexity with regard to cell growth, differentiation, and

matrix turnover (Saminathan et al. 2013). Li et al. (2011, 2013) reported 25 g/cm² as the optimum compressive stress in 3D PDL *in vitro* models. This force level is quite similar to that acquired from animal studies and clinical experience, suggesting that this tissue model is superior to 2D cultures for mimicking the reaction of PDL to orthodontic force (Schwarz 1932). The loading duration used in this study, however, only reached 72 h, which is still short compared to the 10-day clinical tooth movement period, making the prediction of time-dependent gene expression difficult (Proffit et al. 2006). Our tissue model using enhanced culture media permeability was shown by SEM and caspase-3/7 to endure at least 14 days under compressive force up to 35 g/cm² with no excess cell apoptosis. This suggests that our model system could represent a substantial improvement with regard to cell survival and for evaluating gene expression over a more clinically relevant time period.

In the present research, we used compressive stresses of 5, 15, 25 and 35 g/cm², which we believe to be appropriate values of orthodontic force. Caspase-

Fig. 6 Caspase-3 and caspase-7 detection following the application of various compressive stress conditions. In all figures, similar density of green fluorescence was observed among the

in vitro HPdLLTs without any relationship to the compressive load duration or compressive stress intensity

3/7 expression in samples from day 1 to day 14 using compressive stress from 0 to 35 g/cm² showed similar green fluorescent light density, suggesting similar levels of apoptosis regardless of the applied force. According to the surface and cross-sectional SEM observations, cell and extracellular matrix morphology were also similar regardless of compressive stress intensity or duration. These results indicate that cells are viable when subjected to compressive stress levels of at least 35 g/cm² for 14 days, suggesting sufficient cell survival to provide a basis for follow-up gene expression studies.

A primary goal of this study was to observe the osteogenic and osteoclastic gene expression patterns of HPdLLT under various conditions of compressive stress. RANKL is an essential TNF family cytokine for the differentiation of cells from monocyte macrophages to osteoclasts (Anderson et al. 1997; Simonet et al. 1997). It was previously observed that RANKL mRNA expression was significantly increased at compressive stress loads of 25 g/cm² or greater, peaking at 6 h at 25 g/cm² (Li et al. 2011). Another

report also showed that RANKL mRNA expression under 48 h of 2.0 g/cm² compressive stress was significantly higher compared than that at 0 or 4.0 g/cm², and that RANKL mRNA expression increased with the duration of compressive stress (Kim et al. 2009). RANKL is believed to be an essential osteoclastogenesis inducer; therefore, the peak of RANKL expression deduced from the polyline should provide a more expedient representation of compressive stress for osteoclastogenesis (De Araujo et al. 2007). Although these studies showed similar RANKL expression patterns that do not reflect a single linear relationship with compressive stress, they did not determine whether RANKL expression can have another peak. However, it would not be surprising if there were two peaks of RANKL expression. Clinically, light optimal orthodontic force leads to optimal orthodontic tooth movement, and excess heavy force leads to root resorption (Proffit et al. 2006). In an animal experiment, the application of heavy force up-regulated RANKL mRNA and led to root resorption confirmed by H&E staining (Low et al. 2005). In a

Fig. 7 Gene expression of HPdLLTs following application of various compressive stress conditions. The RANKL gene showed a characteristic double peak on day 7 and day 14 at 15 and 35 g/cm² compressive stress. BMP-2 gene showed the double peak only on day 14 at 15 and 35 g/cm². PLAP-1 and ALP showed expression peaks on day 14 at 5 g/cm². OPG and FGF-2 showed similar gene expression, with significantly higher levels on day 14 compared to earlier days. COL1A1 expression on days 7 and 14 was significantly higher than that on days 1 and 3 at all compressive stress levels

cellular experiment, the RANKL expression of the root resorption group was much higher than that in the control (Yamaguchi et al. 2006). Animal studies have indicated that a continuous force of not more than 15–20 g/cm² should be used to safely apply a optimum biological tooth movement; stronger force comprises risky “heavy” that may lead to undesired effects (Schwarz 1932). Hence, the double-peak RANKL up-regulation on day 7 and day 14 at 15 and 35 g/cm² in our results may represent one peak for optimal orthodontic tooth movement, and another for root resorption. Additionally, regarding time course, our results seem to match clinical observations that tooth movement usually occurs in the first 10 days following orthodontic force loading. Our results may mimic not only optimal orthodontic force but also mimic orthodontic force, as up-regulation of RANKL expression was observed at 15 and 35 g/cm², respectively. However, these findings need to be confirmed by additional research.

Li et al. (2011) found that the peak of BMP-2 expression between 0 and 72 h under 25 g/cm² compressive stress appeared at 6 h in their 3D model system, which seems inconsistent with the findings of the present study. We found higher BMP-2 expression on day 3 (about 72 h) than on day 1 (about 24 h) under 25 g/cm² compressive stress. In a 2D periodontal ligament culture system, Mitsui et al. found that until 24 h, compressive stress of 1.0 g/cm² always showed the highest BMP-2 expression within the range of 0–3.0 g/cm² (Mitsui et al. 2006). Although results from 2D and 3D model systems vary, these previous studies and the present investigation found similar trends in both BMP-2 and RANKL expression. BMP-2 is known to play a key role in osteoblast differentiation; it signals cross-communication with RANKL-mediated osteoclastic differentiation and survival, although enhanced RANKL-induced osteoclast survival through BMP-2 is not related to nuclear factor- κ B activation (Itoh et al. 2001). In the present study,

both RANKL and BMP-2 showed a very similar double-peak expression pattern in relation to compressive stress intensity, but RANKL expression showed these characteristic peaks on day 7 and day 14, whereas BMP-2 showed the double peak only on day 14. It can reasonably be assumed that this similarity is related to the direct but limited influence that BMP-2 has on RANKL and suggests that RANKL may be involved in other signaling pathways unrelated to BMP-2 activity.

The expression curves of ALP and PLAP-1 generally showed a gentle increase and decrease on days 1, 3 and 7. Except for day 14, similar expression of PLAP-1 was seen for all previous time points at stress equal to or greater than 15 g/cm², and ALP expression on day 3 was lower than that on day 1 above 5 g/cm². However, a very characteristic trend of gene expression was observed on day 14. Expression levels of both ALP and PLAP-1 genes peaked on day 14 at 5 g/cm² compressive stress. ALP and PLAP-1 mRNA transcripts usually serve as early and late markers of osteogenic activity, respectively (Pinero et al. 1995; Yamada et al. 2007). The expression profiles of these genes in our study indicate that the osteogenic potential may be highest under 5 g/cm² compressive stress.

FGF family proteins are involved in activities such as wound healing, angiogenesis, and endocrine signaling. FGF-2 was found to enhance the proliferation of HPdLFs in a concentration-dependent manner (Hidaka et al. 2012), and the PDL compressive stress study of Li et al. (2011) indicated that FGF-2 was upregulated at 6, 24 and 72 h. In the present study, however, FGF-2 was down-regulated rather than upregulated on day 3 (72 h) compared to day 1 under 25 g/cm² compressive stress. However, according to our overall results, expression of FGF-2 was upregulated over time, indicating an increase in HPdLF proliferation. The enhanced expression of FGF-2 and associated increases in cell proliferation may counteract the cell turnover process, which would normally be expected to become faster because of the lack of living space, oxygen, or nutrition, during long-term culture in the 3D matrix (Xiao and Tsutsui 2013).

OPG is a soluble decoy receptor for RANKL that serves as a negative regulator of osteoclastic differentiation and function induced by RANKL (Simonet et al. 1997). OPG expression was not substantially affected by mechanical compressive stress in our

study. The 3D periodontal ligament model of Li et al. (2011) indicated that the expression of OPG increased after 24 and 72 h under 25 g/cm² compressive stress. However, this may not have resulted from inherent instability of the model. Studies using two dimensional (2D) periodontal ligament culture systems also showed similar trends. Kim et al. (2009) showed that under 0–4 g/cm² compressive stress, expression of OPG did not significantly change until 48 h, and Nakajima et al. (2008) indicated that under 0–4 g/cm² compressive stress, OPG expression increased with longer compressive stress durations.

Collagen accounts for approximately 30 % of the total body protein and is the most abundant protein among the entire extracellular matrix (Byers 2000). Although PDL is known to contain all three types of collagen, fibril-forming type I collagen (COL1) dominates. This type of collagen is reported to be affected by mechanical stress (Sodek and Limeback 1979), and COL1 expression was gradually upregulated in relation to compression load duration in our study. COL1 expression on days 7 and 14 was significantly higher than that at days 1 and 3 at all compressive stress levels. This trend is similar to that reported by Nemoto et al. (2010), who found upregulated COL1 expression after 3 days of stimulation using mechanical pressure.

Cell morphology and apoptosis revealed that this model could endure orthodontic force of up to 35 g/cm² for 14 days. The peaks of gene expression for PLAP-1 and ALP were observed on day 14 at 5 g/cm². A double-peak gene expression pattern was also observed at 15 and 35 g/cm² for RANKL and BMP-2 on day 7 and day 14. The present study found that the reaction and gene expression of human PDL in response to orthodontic force can be mimicked well by applying compressive stress in this in vitro HPdLLT model system. Further investigations should be conducted on the biochemical and biophysical reaction to orthodontic force, including both compression and tension forces, to provide additional clues regarding the reaction of PDL tissue to orthodontic stress.

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Conflict of interest The authors confirm that there is no conflict of interest.

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