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

Wen Liao [a*], Masahiro Okada [b], Kaoru Inami [c], Yoshiya Hashimoto [b*], Naoyuki Matsumoto [c]

a Graduate School of Dentistry, Department of Orthodontics, Osaka Dental University, 8-1 Kuzuha-hanazono-cho, Hirakata-shi, Osaka 573-1121, Japan
b Department of Biomaterials, Osaka Dental University, 8-1 Kuzuha-hanazono-cho, Hirakata-shi, Osaka 573-1121, Japan
c Department of Orthodontics, Osaka Dental University, 8-1 Kuzuha-hanazono-cho, Hirakata-shi, Osaka 573-1121, Japan

**Corresponding authors.*
Address: Department of Orthodontics, Osaka Dental University, 8-1 Kuzuha-hanazono-cho, Hirakata-shi, Osaka 573-1121, Japan. Tel: +81-72-864-3078. E-mail address: liaowenssw@gmail.com (W. Liao)
Address: Department of Biomaterials, Osaka Dental University, 8-1 Kuzuha-hanazono-cho, Hirakata-shi, Osaka 573-1121, Japan. Tel: +81-72-864-3056. E-mail address: yoshiya@cc.osaka-dent.ac.jp (Y. Hashimoto)

Abstract

This study investigated cell survival and gene expression under various compressive stress conditions mimicking orthodontic force using a newly developed *in vitro* model of human periodontal ligament-like tissue (HPdLLT). The HPdLLT was developed by three-dimensional (3D) culturing of human periodontal ligament fibroblasts (HPdLFs) in a porous poly-$_L$-lactide (PLLA) matrix with 3-fold greater culture media permeability by hydrophilic modification. *In vitro* HPdLLTs in experimental groups were subjected to 5, 15, 25, and 35 g/cm^2 compressive stress for 1, 3, 7, or 14 days, and 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 (SEM) and caspase-3/7 detection. Real-time PCR was also performed for seven osteogenic and osteoclastic genes. Similar extracellular matrix and spindle-shaped cells were observed inside or on the surface of each *in vitro* HPdLLT, with no relation to compressive stress duration or intensity. Similar caspase-3/7 activity indicating comparable apoptosis levels was also observed in all samples. RANKL and BMP-2 genes showed a characteristic "double-peak" expression at 15 and 35 g/cm^2 on day 14, ALP and PLAP-1 expression peaked at 5 g/cm^2 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 human PDL in response to orthodontic force, and this work expanded our knowledge on the effects of compressive stress on human PDL tissue.

Key Words

Human periodontal ligament-like tissue
Orthodontic Force
Gene Expression
Cell living condition
Compressive Stress

Introduction

Human periodontal ligament (PDL) tissue is a flexible and well-organized connective tissue, providing the support structure of teeth. PDL connects the tooth cementum to the alveolar bone and also provides support, protection, and the sense 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, which has been investigated in many studies of 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 gained 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), 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. 2013; Li et al. 2011; 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 culturing human periodontal ligament fibroblasts in a porous poly-$_{L}$-lactide matrix. This matrix was hydrophilically modified using ammonia solution, allowing for 3-fold 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 PLAP-1, ALP, FGF-2, and COL1 were upregulated. This suggested that culturing HPdLFs in a hydrophilically modified PLLA matrix was 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 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 casted 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 and 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's Medium, along with antibiotic/antimycotic (Nacalai Tesque). Cells were cultured at 37 ºC in a 5% CO_2 incubator. 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 HPdLLT (Kim et al. 2013; Li et al. 2011; Nakajima et al. 2008). HPdLLTs were continuously compressed using the uniform compression method shown in Fig. 2. In brief, each *in vitro* HPdLLT was cultured in one well of a 24-well plate (BD Falcon, Franklin lakes, NJ, USA). A 15-mm-diameter layer of round micro cover glass (Matsunami, Kishiwada, Japan) was placed over each *in vitro* HPdLLT. Metal weights were placed on the cover glass in order to apply compressive stress to the *in vitro* HPdLLTs. In the experimental group, *in vitro* HPdLLTs were subjected to 5 g/cm^2, 15 g/cm^2, 25 g/cm^2 or 35 g/cm^2 of compressive stress for 1, 3, 7, or 14 days, respectively. The control group (0 g/cm^2) was cultured for 1, 3, 7, or 14 days and subjected to no 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-section images (×250; ×1000) of all samples were obtained using a scanning electron microscope (S-4000; NEC, Tokyo, Japan) at 3 kV.

Caspase-3/7 detection

Observation of caspase-3/7 activity was performed using a fluorescent staining kit (CellEvent™ Caspase-3/7 Detection Kit; Life Technologies, Tokyo, Japan). All procedures were carried out according to the manufacturer's recommended protocol. Briefly, samples were first incubated with reagent in a 5% CO_2 incubator at 37 ºC 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 synthesizing kit (Transcriptor Universal cDNA Master; Roche Diagnostics, Mannheim, Germany). Real-time polymerase chain reaction (RT-PCR) was carried out with a probe kit (Universal ProbeLibrary Set, Human, Roche Diagnostics), master mix (FastStart Universal 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, USA). Sequences of order-made primers used in PCR were listed in Table 1. All RNA isolation, cDNA synthesizing and RT-PCR were carried out according to manufactures recommended protocols.

Statistical analysis

For all experiments, values are reported as mean ± SD (standard deviation) for triplicate cell cultures. Differences were evaluated by analysis of variance (ANOVA) with Tukey–Kramer correction 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

SEM images of the *in vitro* HPdLLT samples under constant compressive stress or controls were obtained on days 1, 3, 7, and 14. Fig. 3 shows the SEM images of the surfaces of 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. Cells connected clearly with each other, and the extracellular matrix was also observed. In other *in vitro* HPdLLTs (0 g/cm^2 on day 3; arrow B) the extracellular matrix obscured HPdLFs. In each case, *in vitro* HPdLLTs were considered viable and mature. Cross-section 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 caspase-3/7 activity of *in vitro* HPdLLTs subjected to compressive stress and controls were obtained on days 1, 3, 7, and 14. Fig. 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, most significant differences in gene expression were observed on day 14 compared to previous durations at 15 g/cm^2, 25 g/cm^2 and 35 g/cm^2 compressive stress intensities. 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 significantly higher compared to 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 the 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 higher. Type I collagen (COL1) showed relatively stable gene expression regardless of the intensity of compressive stress. There were no significant differences between the day 7 and day 14 expression of COL1, 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 regulating 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 that there were no differences in cell living conditions between various compressive force intensities and durations.

The clinical value of optimum orthodontic force was previously reported to be 10 g to 120 g (Proffit et al. 2006), or not greater than the pressure in the blood capillaries, which is about 20 to 26 g/cm^2 (Schwarz 1932). In studies using 2D cultured cells, however, Mitsui et al. and Nakajima et al. reported well-functioning light and heavy orthodontic forces of only 0.5 g/cm^2 and 4 g/cm^2, respectively, although their

loading duration was no longer than 24 h. (Mitsui et al. 2006; Nakajima et al. 2008). 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 is unlikely to mimic the time-dependent gene expression patterns that occur over a 10 day period, so 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. reported 25 g/cm^2 as the optimum compressive stress in 3D PDL *in vitro* models (Li et al. 2013; Li et al. 2011). 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/cm^2 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/cm^2, which we believe to be appropriate values of orthodontic force. Caspase-3/7 expression in samples from day 1 to day 14 using compressive stress from 0 g/cm^2 to 35 g/cm^2 showed similar green fluorescent light density, suggesting similar levels of apoptosis regardless of the applied force. According to the surface and cross-section SEM observations, cell and extracellular matrix morphology were also similar without regard to compressive stress intensity or duration. These results indicate that cells are viable when subjected to compressive stress levels of at least 35 g/cm^2 for 14 days, suggesting sufficient cell survival to provide the basis of follow-up gene expression studies.

A primary goal of this study was to observe 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). In a report, it was observed that RANKL mRNA expression significantly increased at compressive stress loads of 25 g/cm^2 or greater, peaking on 6 h at 25 g/cm^2 (Li et al. 2011). Another report also showed that RANKL mRNA expression under 48 h of 2.0 g/cm^2 compressive stress was significantly higher compared to 0 g/cm^2 or 4.0 g/cm^2, 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 be a more expedient 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 to

compressive stress, they did not prove whether RANKL expression can have another peak. However, it is not surprising if there were two peaks of RANKL expression. Clinically, light optimal orthodontic force lead to optimal orthodontic tooth movement, excess heavy force lead to root resorption(Proffit et al. 2006). In an animal experiment, the application of heavy force up-regulated RANKL mRNA expression and led to root resorption confirmed by H&E staining (Low et al. 2005). In a cellular experiment, the RANKL expression of root resorption group was much greater than the control (Yamaguchi et al. 2006). Animal studies have indicated that continuous force of not more than 15 to 20 g/cm^2 should be used to apply a biological optimum tooth movement safely and force heavier than it are risky "heavy" force which may lead to undesired effects(Schwarz 1932). Hence, the "double-peak" RANKL up-regulation on day 7 and day 14 at 15 g/cm^2 and 35 g/cm^2 in our result can be one peak for optimal orthodontic tooth movement, and another for root resorption. What's more, on the time-dependent observation, our results seem to match clinical observations that tooth movement usually occurs in the first 10 days following orthodontic force loading. Our results may not only mimic optimal orthodontic force, but may also mimic heavy orthodontic force. They up-regulated RANKL expression on15 g/cm^2 and 35 g/cm^2, respectively. However, these findings needs to be confirmed by additional research.

Li et al. found that the peak of BMP-2 expression between 0 h and 72 h under 25 g/cm^2 compressive stress appeared at 6 h in their 3D model system (Li et al. 2011), which seems inconsistent with the present study. We found higher BMP-2 expression on day 3 (about 72 h) compared to day 1 (about 24 h) under 25 g/cm^2 compressive stress. In a 2D periodontal ligament culture system, Mitsui et al. found that up until 24 h, compressive stress of 1.0 g/cm^2 always showed the highest BMP-2 expression within the range of 0 g/cm^2 to 3.0 g/cm^2 (Mitsui et al. 2006). Although results from the 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 with nuclear factor-kB 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 while 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 rise and fall on days 1, 3, and 7. Except for day 14, similar expression of PALP-1 was seen for all previous time points at stress equal to or greater than 15 g/cm^2, and ALP expression on day 3 was lower than that on day 1 above 5 g/cm^2. 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/cm^2 compressive stress. ALP and PLAP-1 mRNA

usually serves 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 osteogenic potential might be highest under 5 g/cm^2 compressive stress.

FGF family proteins are involved in activities such as wound healing, angiogenesis and endocrine signaling pathways. FGF-2 was found to enhance the proliferation of HPdLFs in a dose-dependent manner (Hidaka et al. 2012), and the PDL compressive stress study by Li et al. indicated that FGF-2 expression was upregulated at 6 h, 24 h, and 72 h (Li et al. 2011). 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/cm^2 compressive stress. However, according to our overall results, the expression of FGF-2 was upregulated in a time-dependent manner, 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 due to lack of living space, oxygen, or nutrition on long-term culturing 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. indicated that the expression of OPG increased after 24 h and 72 h under 25 g/cm^2 compressive stress (Li et al. 2011). However, this may not be due to an inherent instability of the model. Studies using two dimensional (2D) periodontal ligament culture systems also showed similar trends. Kim et al. showed that under 0 to 4 g/cm^2 compressive stress, the expression of OPG showed no statistically significant change until 48 h (Kim et al. 2009), and results from Nakajima et al. indicated that under 0 to 4 g/cm^2 compressive stress, OPG expression increased with longer compressive stress durations (Nakajima et al. 2008).

Collagen accounts for about 30% of 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 of days 1 and 3 at all compressive stress levels. This trend is similar to that published by Nemoto et al., who found upregulated COL1 expression after 3 days of stimulation using mechanical pressure (Nemoto et al. 2010).

Cell morphology and apoptosis revealed that this model could endure orthodontic force up to 35 g/cm^2 for 14 days. In the osteogenetic genes, the peaks of gene expression for PLAP-1 and ALP were observed on day 14 at 5 g/cm^2. A "double-peak" gene expression pattern was also observed at 15 g/cm^2 and 35 g/cm^2 for RANKL and BMP-2 on day 7 and/or day 14. The present study revealed 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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The authors confirm that there is no conflict of interests.

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Figures and Table Legends

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

Fig. 1. Microscope image (A) of the poly-(L-lactic acid) (PLLA) matrix. Scanning electron microscopic image of PLLA matrix without seeded cells (B) and with cells grown for 14 days (C).

Fig. 2. Method used to apply compression stress to mimic orthodontic force on the PDL. Pre-cultured *in vitro* HPdLLTs were compressed continuously 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 mimic orthodontic force and the *in vitro* HPdLLTs mimic the PDL tissue.

Fig. 3. Surface SEM observation of *in vitro* HPdLLTs (×100) after the 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 relation to compressive load duration or

compressive stress intensity.

Fig. 4. Cross-section SEM observation of *in vitro* HPdLLTs (×1000) after the application of various compressive stress conditions. In all figures, similar filamentous extracellular matrix and spindle-shaped cells can be observed inside each *in vitro* HPdLLT with no relation to compressive load duration or compressive stress intensity. This figure is a local amplification of Fig.5 (×250), which shows the extracellular matrix and spindle-shaped cells observed from the surface to the rear side of each *in vitro* HPdLLT.

Fig. 5. Cross-section SEM images of HPdLLTs (×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.

Fig. 6. Caspase-3 and caspase-7 detection following the application of various compressive stress conditions. In all figures, similar density of green fluorescent cells observed inside the *in vitro* HPdLLT without relation to the compressive load duration or compressive stress intensity.

Fig. 7. Gene expression of the HPdLLTs following the application of various compressive stress conditions. RANKL gene showed a characteristic "double-peak" on day 7 and day 14 at 15 g/cm^2 and 35 g/cm^2 compressive stress. BMP-2 gene showed the "double-peak" only on day 14 at 15 g/cm^2 and 35 g/cm^2. PLAP-1 and ALP showed expression peaks on day 14 at 5 g/cm^2. OPG and FGF-2 showed similar gene expression, with significantly higher levels on day 14 compared to earlier days. COL1 expression on days 7 and 14 was significantly higher than that of days 1 and 3 at all compressive stress levels.