

Effects of Continuous and Interrupted Forces on Gene Transcription in Periodontal Ligament Cells in Vitro

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Abstract—The biological mechanisms of tooth movement are based on the response of periodontal tissues to mechanical forces. The final result of these responses is remodeling of the extracellular matrix. Tissue reactions may vary depending upon the type, magnitude and duration of the applied forces. The purpose of the present study was to analyze the effects of centrifugal force on the transcription of collagen type-I (Col-I), matrix metalloproteinase-1 (MMP-1), and tissue inhibitor of metalloproteinase-1 (TIMP-1) genes in human periodontal ligament (PDL) fibroblasts. Human fibroblasts obtained from the PDL were cultured and subjected to centrifugal forces (36.3 g/cm²) for 30, 60 and 90 min continuously. This was also carried out interruptedly, three times for 30 min and six times for 15 min. The mRNAs encoding for Col-I, MMP-1, and TIMP-1 were quantified using RT-PCR. The mRNA levels of Col-I and MMP-1 were increased when continuous force was applied for 30 min and 60 min respectively. The interrupted force had almost no effect on Col-I, MMP-1 and TIMP-1 genes. These results indicate that continuous forces may have a greater effect in inducing gene expression during the remodeling process of PDL compared to interrupted forces with short rest periods.

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Introduction

Orthodontic tooth movement (OTM) is a mechanotransduction event involving a complex feedback mechanism. Numerous transcriptional factors are involved in the differentiation, proliferation and function of different cell types, which eventually lead to mechanically induced remodeling. Tissue response to OTM is no longer described as an inflammatory process in modern orthodontic literature but it is best regarded as “an exaggerated form of normal physiological turnover combined with foci of tissue repair” (1).

The remodeling process of periodontal tissue involves modification of the extracellular matrix (ECM).

Collagen fibers, the principal components of the ECM, are responsible for the coherence of connective tissues. Tissue remodeling during OTM requires the degradation of these proteins to allow for tissue resorption. ECM remodeling is mediated by a class of zinc dependent enzymes, matrix metalloproteinases (MMPs) which are able to degrade most proteins of the ECM. MMPs are counteracted by regulatory factors such as tissue inhibitors of metalloproteinases (TIMPs) (2). All active MMPs are inhibited by TIMPs allowing control of connective tissue breakdown (3,4). The rate of ECM remodeling which is determined by the balance between MMPs and TIMPs is important for maintaining the integrity of healthy tissues, in physiologic conditions

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such as OTM and also in the pathologic situations such as rheumatoid arthritis, cancer, periodontitis (5) and in malformations such as cleft lip and palate (6).

Because of the complexity of the *in vivo* environment, investigators have relied on *in vitro* methods to study the mechanisms that are involved in transforming forces into biochemical signals by application of controlled mechanical input to cell culture systems (7). *In vitro* studies have been used along with *in vivo* OTM studies to demonstrate the direct effect of mechanical strain on gene expression of human PDL cells. Cyclical forces have been shown to be more effective than continuous forces in stimulating collagenase production, while secretion of the collagenase inhibitor was unaffected by either form of mechanical stimulation (8). Continuous tensile strain applied to PDL cells stimulated the expression of MMP-1, MMP-2, TIMP-1 and TIMP-2 (9). Centrifugal force has been reported to up-regulate mRNA levels for MMP-1 with little or no effect on collagen type-I (Col-I) or TIMP expression (10), while an equibiaxial tensile strain applied at low magnitudes, suppressed the mRNA expression of MMP-1 and MMP-3 (11). However, the exact mechanisms by which biomechanical forces lead to biological response are not fully understood.

For the present study, we propose that applying different types of strains to PDL cells during orthodontic tooth movement would influence the differential expression of genes involved in alveolar bone remodeling. We therefore applied continuous and intermittent forces to human PDL cells in monolayer culture and quantified the expression of collagen type-I, matrix metalloproteinase-1, and tissue inhibitor-1 using polymerase chain-reaction (PCR) analysis.

Materials and Methods

Preparation of human PDL cells

Human PDL fibroblasts were isolated from the ligament tissues of periodontally healthy, non-carious premolar teeth which were extracted from three donors with their informed consent (approved by the ethical committee of Tehran University of Medical Sciences), according to the method by Somerman *et al.* (12). The patients were asked to rinse their mouth with Chlorhexidine 0.2% mouthwash prior to extraction of teeth to reduce contamination. The teeth were immediately immersed in Dulbecco's modified essential medium (DMEM) solution that was supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin; (Gibco-BRL Grand Island, NY, USA)

then delivered to the cell culture lab. After removing any remaining gingival tissue from the cervical portions and rinsing the teeth several times with phosphate-buffered saline (PBS), The PDL tissue of the middle third was scraped with surgical blades and transferred to a 25 cm² culture flask (Nunc, Denmark) containing 3ml culture medium which was then incubated at 37°C in the presence of 5% CO₂. The PDL tissue of the cervical and apical portions of the root were not used to avoid contamination of the culture with gingival or pulp cells. After ten days the cells were grown out of explant. When the cells had reached confluence, they were detached with 0.05% trypsin in PBS and subcultured in 75 cm² culture flasks (Nunc, Denmark). The flasks were discarded after each subculture to avoid contamination. Experiments were carried out with cells from third to fifth passages (p3-p5).

Application of centrifugal force

2×10⁵ cells were cultured in each well of a 6 well plate. The culture medium was changed the next day. Centrifugal force was applied 48 hours following culture. At this stage the cells were 80-90% confluent.

Cell culture plates were centrifuged at 118 g and 37°C in five different conditions to mimic continuous and interrupted force applications. Cells were centrifuged for 30, 60 and 90 min continuously. We also tested 90 min force application in two interrupted condition; three times for 30 min and six times for 15 min with 5 min intervals in order to compare with continuous conditions. All force application was performed by a Hettich Universal 32R, equipped with a 1645 horizontal plate rotor (Andreas Hettich GmbH & Co. KG Tuttlingen, Germany). The centrifugal force applied to cells had a magnitude of 36.3 g/cm². These models induce vertical shear stress and are based on application of a constant pressure centrifugal force which resembles clinical orthodontic force (13). The calculation of force is based on the following equation:

$$P = (m \times r \times \text{r.p.m.}^2 \times \pi^2) / (A \times 9.8 \times 900)$$

Where P=kg pressure per cm² of cells, *m*=mass of medium, (0.003 kg), *r*=radius (0.106 m), r.p.m.=revolution/min (1000), *A*=area of contact between medium and cells (9.6 cm²).

Isolation of RNA

In order to evaluate the gene expression, twenty four hours after application of centrifugal force, the control and centrifuged cells of each well were washed with cold PBS and homogenized with 0.5 mL TriPure Isolation Reagent (Roche Applied Science, Mannheim,

Germany). The total RNA was purified according to the manufacturer's protocol. Briefly, the lysate was mixed with 0.1 mL chloroform and centrifuged at $12,000 \times g$ for 15 minutes at 4°C . The colorless upper aqueous phase was transferred to a new microtube. The RNA was precipitated from this phase by adding 0.25 mL isopropanol and centrifuging at $12,000 \times g$ for 10 minutes at 4°C . The pellet was washed with 0.5 mL 75% ethanol and centrifuged at $7500 \times g$ for 5 minutes at 4°C . The RNA pellet was resuspended and dissolved in diethylpyrocarbonate (DEPC)-treated RNase-free water and stored at -80°C . The total RNA was quantified spectrophotometrically using $\text{OD}_{260\text{nm}}$ prior to cDNA synthesis.

Semi-quantitative RT-PCR analysis

The mRNA levels of β -actin, Col-I, MMP-1, and TIMP-1 were measured with semi-quantitative RT-PCR analysis. cDNA was synthesized from 2.0 μg of total RNA in 20 μL of reaction buffer composed of 2 mM dNTPs, 50 U ribonuclease inhibitor, random hexamere and 200 U of Murine Leukemia Virus (MLV) reverse transcriptase (all from Fermentas, Ukraine).

Oligonucleotide primer sequences for the PCR reaction were: β -actin (forward, 5'-GATGATGATA TCGCCGCGCT-3'; reverse, 5'-CTTCTCGCGGTTGGCCTTGG-3') (14), Col-I (forward, 5'-CTGGCAAAGAAGGCGGCAAA-3'; reverse, 5'-CTCACCACGATCACCA CTCT-3'), MMP-1 (forward, 5'-TGGGAGCAAACACATCTGA-3'; reverse, 5'-ATCACTTCTC CCCGAATCGT-3') (10) and TIMP-1 (forward, 5'-CCCCTGGCTTCTGGCAT CCTGTTG-3'; reverse, 5'-CTCCATGGCGGGGGTGT

AGACGAA-3'). PCR primers were designed for TIMP-1 cDNA with the Primer Select program of DNASTar software (DNASTAR Inc., USA). PCR was performed using a program consisting of denaturing at 95°C for 3 min, annealing (β -actin and collagen 62°C , MMP 48°C and TIMP 52°C , for 30 sec), extension at 72°C for 45 sec, for 35 cycles followed by an extension step at 72°C for 10 min. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. The intensity of each band was quantified by means of Scion Image software (Scion Corporation, Frederick, MD, USA) and expressed relative to the intensity of the band obtained with human beta-actin gene as an internal control.

Statistical analysis

The data are given as means \pm standard deviation. Results are presented as a ratio of the amount of each Col-I, MMP-1 and TIMP-1 mRNA by β -actin mRNA. The normalized mRNA levels for each gene in mechanically stimulated cells were compared with those of control cells by paired *t* test. P values less than 0.05 were considered significant.

Results

To evaluate the effect of continuous and interrupted forces on gene expression of human PDL fibroblasts, cell culture plates were centrifuged for 30, 60, and 90 min continuously, and 3×30 min and 6×15 min interruptedly. mRNA levels were determined 24 hours after application of centrifugal force (Figure 1).

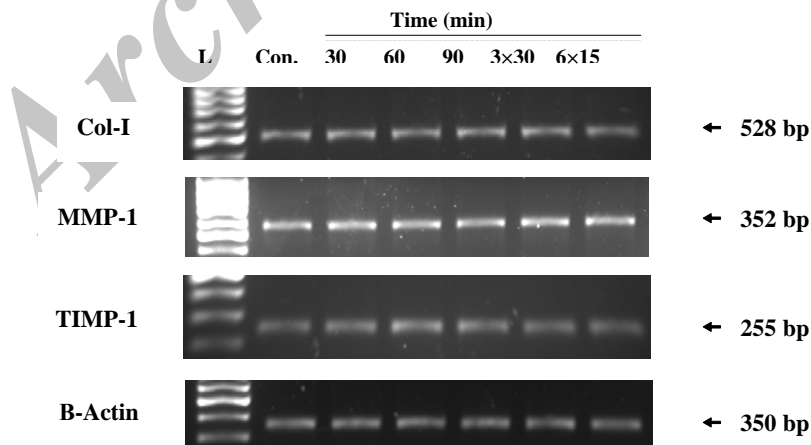


Figure 1. Results of semi-quantitative RT-PCR analysis. The mRNA expression of Col-I, MMP-1, TIMP-1 and β -actin in human PDL fibroblasts were evaluated under continuous and interrupted force. Cells were cultured in 6 well plates and submitted to centrifugal force continuously for 30, 60 and 90 min, and interruptedly for 3×30 and 6×15 min with 5 min interval between load cycles. Total RNA was extracted and amplified with RT-PCR using specific primers for each gene. The RT-PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining.

Table 1. mRNA levels encoding for the Col-I, MMP-1 and TIMP-1 in control and mechanically stressed cells after normalization to β -Actin.

Gene	Control	30 min	60 min	90 min	3×30 min	6×15 min
Col-I	0.83±0.04	1.13±0.03*	1.05±0.21	0.94±0.21	1.08±0.26	1.05±0.24
MMP-1	1.24±0.18	1.78±0.34	1.81±0.16*	1.48±0.27	1.45±0.19	1.33±0.59
TIMP-1	0.89±0.34	1.21±0.54	1.20±0.43	1.20±0.48	1.18±0.53	1.09±0.69

*Significant difference ($P<0.05$) in centrifuged cells when compared to control cells.

The appropriate range of forces have been chosen in conditions with at least 90% viability using trypan blue dye exclusion test compared to control. The levels of mRNAs encoding for Col-I, MMP-1, and TIMP-1 in different durations and modes of force applications are shown in Table 1. The level of β -actin gene was almost the same as the amount of control sample at different durations of continuous and interrupted force applications.

Centrifugation stimulated expression of the collagen gene by approximately 1.4 fold greater than untreated control cells at 30 min ($P<0.01$). The stimulatory effect decreased with the increase in the centrifugation time up to 90 min. At 3×30 min, the collagen level was greater

than 60 and less than 30 min of continuous force application. At 6×15 min, the level of collagen was almost equal to the level of 60 min continuous force application.

The expression of MMP-1 gene after 30 min of centrifugation was 1.4 fold greater than the control culture but the difference was not significant. Up-regulation of the MMP-1 gene increased to 1.5 fold of the control sample at 60 min ($P<0.05$). The amount of MMP-1 expression at 90 min continuous force and 3×30 min interrupted force were almost the same amount with some reduction at 6×15 min of interrupted force application which was still greater than the control level.

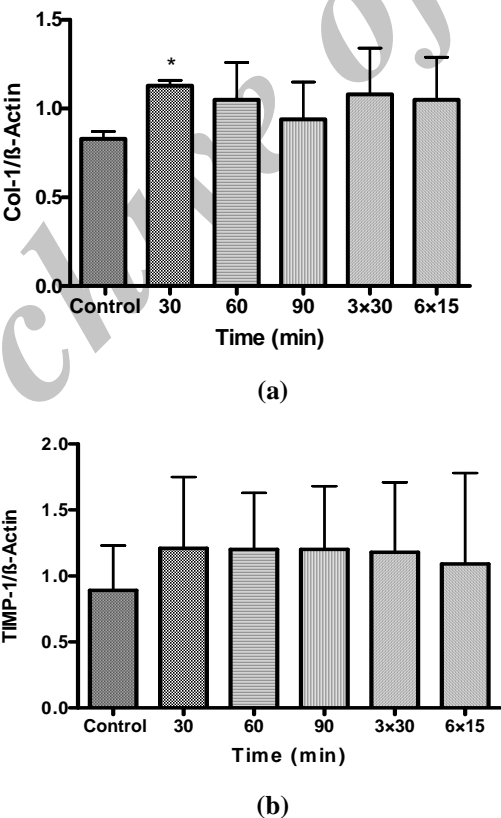


Figure 2. mRNA expression of Col-I, MMP-1, TIMP-1 and β -actin in human PDL fibroblasts. The results of RT-PCR were subjected to densitometric analysis and calculated as ratio to β -actin. Values obtained from three independent experiments ($n=3$) are expressed as mean \pm SD. * $P<0.05$.

The amount of TIMP gene increased 1.4 fold compared to the untreated control sample at 30 min of force application, which was not statistically significant. However, continuous application of centrifugal force for 60 and 90 min did not induce TIMP-1 expression above those present in untreated control cells. The up-regulation of TIMP gene expression decreased at 3×30 min of interrupted force application and reached almost 1.2 folds greater than the control level at 6×15 min (Figure 1).

Discussion

In orthodontics treatment, rational is based on the capacity of the alveolar bone to remodel following application of controlled force systems. Fibroblast cells which are the most common cell type in PDL, have osteoblastic-like phenotypic characteristics and high basal alkaline phosphatase activity (15). These cells are the prominent mediators that sense mechanical stimuli, transmit the signals intracellularly via transmembrane receptors and convert them into biomechanical responses as a result of differential gene expression (16). Many *in vivo* studies have shown the modulation in the expression of different genes responsible for the turnover of periodontal tissues, following application of mechanical force (17-21).

During OTM, the PDL may experience compressive, tensile, shear or a combination of stress types. *In vitro* methods have expanded our knowledge regarding the effects of mechanical strain on expression of different genes that are involved in the remodeling of PDL at both message and protein levels. The differences in the results reported may be partly due to the different types of stresses applied to the cells *in vitro*. It remains unclear as to what extent cells distinguish between different types of mechanical stimulation. The majority of studies have focused on the effects of tensile strain on cultured human PDL fibroblasts (22), and have mostly subjected these cells to different percentage of elongation by use of a Flexercell Strain Unit (16). In some studies, compressive force has been used to investigate the response of cells to mechanical stimulation (23). During OTM, the PDL experiences more than a tensile force at the tension side and a compressive force at the pressure side. Similar to the situation observed in craniofacial sutures (24), shear stress may arise in PDL during the application of tensile or compressive forces. This may be partly due to the fibrous connective tissue structure that occupies the periodontal space as well as the uneven surface of the alveolar bone. Compared to tensile and

compressive stress, the shear forces act over an area which is in line with the forces. Centrifugation has been used as another approach for mechanostimulation and is based on applying fluid shear stress to cell culture flasks. Both mechanoreception and response are influenced by this phenomenon (7).

The centrifuge rotor utilized in this experiment was able to apply shear forces to cultured cells.

Since cell damage is an important issue in the study of cell response to applied mechanical loads, we measured the cell viability in all conditions. The appropriate ranges of forces have been chosen by detecting at least 90 %viability using the trypan blue dye exclusion test. Thus, we ensured that the range of the mechanical forces applied both continuously and interruptedly did not damage the cultured cells.

Semi-quantitative RT-PCR was performed to study the expression levels of mRNA for target genes. Short time mechanical strain for 30 and 60 min were effective in stimulating Col-I and MMP-1 genes respectively, but had no effect on the expression of the TIMP-1 gene. In a previous experiment (10), MMP-1 gene was upregulated following application of centrifugal force for 30 min which further confirms that mechanical strain can directly affect MMP-1 induction. Although the Col-I gene expression increased in the above-mentioned study, unlike the present investigation, the changes were not significant. Induction of Col-I and MMP-1 genes in this study suggests that mechanical force can directly effect the expression of these genes. The minor conflicts observed in the findings of these two studies are probably due to differences in the character of the applied centrifugal force. In neither of these studies, application of continuous force for 90 min affected the expression of Col-I and MMP-1 genes.

The application of centrifugal force was unable to affect the expression of TIMP-1 gene in the present study which corresponds with the findings of the previous study (10). Our finding, that mechanical force is incapable to induce the expression of TIMP-1 gene may indicate that factors other than mechanical strain, such as growth factors and cytokines are mediating the release of this inhibitory enzyme (5). However, since several polymorphisms of MMPs and TIMPs may exist, the response of cells from different origins to the same stimulus should be carefully interpreted (3,4).

Frequency and duration of the mechanical signals are important characteristics that can influence the biological response to the applied force. Timely

reactivation of mechanical stress increased the levels of mediators responsible for OTM (25). Short duration of extremely low-level mechanical signals, caused an increase in bone mass in the weight-bearing skeleton of young adult females with low bone mineral density (BMD) (26). In this study, we were not able to show gene upregulation following application of interrupted forces. PDL fibroblasts exposed to intermittent biaxial deformation exhibited an increase in type I collagen (27). Our findings did not show any alterations in the expression of Col-I by interrupted mechanical forces. The different findings observed in these studies are probably due to the different types of forces applied to cells.

Intermittent forces are generally considered to be more suitable for OTM, because the periodontal tissue can repair itself following a period of turnover. In a previous study, both continuous and cyclical tensile forces caused a statistically significant increase in collagenase production, but the amount of collagenase production was higher when the cells were under cyclical compared to continuous deformation (8). Bone cells become increasingly less sensitive to uninterrupted load cycles (28). Reapplication of force in the present study seems to significantly affect gene expression which indicates that frequent activation of short duration forces may actually inhibit the remodeling process. On the other hand, it has been reported that a couple of hours is required for cells to restore mechanosensitivity (29). This study showed an upregulation of both Col-I and MMP-1 genes following stimulation of fibroblasts with continuous centrifugal force. It is reasonable to suggest that in this type of force system, the cells are less sensitive to the applied force at the beginning of the next cycle because the stimulatory mechanism diminishes after the rest periods which exist between the activations. The results of a previous study showed a change in the orientation of microtubules of PDL cells in response to cyclic stretching, which could represent a self-protection mechanism (30). Further experiments with different durations of rest periods between load cycles are required to further clarify this phenomenon.

In summary, the results of this semi-quantitative analysis shows that human PDL cells exposed to mechanical force can express genes that are involved in bone remodeling. However, further investigations are required to demonstrate the extent to which the message is translated into proteins. Transduction of mechanical stress into tissue remodeling is the result of a complex interaction between the cytoskeleton, various autocrine, and paracrine factors and effector molecules.

Identification of the different mediators of this mechanotransduction system may facilitate the introduction of novel approaches to selectively control bone remodeling during OTM. Real-time PCR microarray analysis, which is an advanced technique, is a more advantageous method since it gives us the opportunity to simultaneously screen a large number of genes under identical experimental conditions.

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