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Original article

Early responses of human periodontal ligament fibroblasts to cyclic and static mechanical stretching

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Summary

Objective: To compare the mechanotransduction caused by cyclic and static mechanical strains in human periodontal ligament fibroblasts (hPDLFs) cultured under identical conditions.
Materials and methods: hPDLFs, originating from the same donors, were exposed either to cyclic or to static tensile strain using specially designed devices and under identical culture conditions. Activation of all members of mitogen-activated protein kinases (MAPKs) was monitored by western immunoblot analysis. Expression levels of immediate/early genes *c-fos* and *c-jun* were assessed with quantitative real-time polymerase chain reaction.
Results: Time course experiments revealed that both types of stresses activate the three members of MAPK, that is ERK, p38, and JNK, with cyclic stress exhibiting a slightly more extended activation. Further downstream, both stresses upregulate the immediate/early genes *c-fos* and *c-jun*, encoding components of the activator protein-1 (AP-1), a key transcription factor in osteoblastic differentiation; again cyclic strain provokes a more intense upregulation. Six hours after the application of both strains, MAPK activation and gene expression return to basal levels. Finally, cells exposed to cyclic stress for longer periods are distributed approximately perpendicular to the axis of the applied strain, whereas cells exposed to static loading remain in a random orientation in culture.
Conclusion: The findings of the present study indicate similar, although not identical, immediate/early responses of hPDLs to cyclic and static stretching, with cyclic strain provoking a more intense adaptive response of these cells to mechanical deformation.

Introduction

Periodontal ligament (PDL) is a highly specialized, connective tissue located between the tooth root and the alveolar bone. The predominant cell type in this tissue is PDL fibroblasts (PDLFs), which are capable for an osteoblastic differentiation, thus contributing to several processes including repair and regeneration, as well as remodelling of the surrounding hard tissue (1).The osteoblastic differentiation of PDLF can be achieved in response to a variety of extracellular stimuli, including mechanical strain (2–4). Specifically, they are subjected to both cyclic strains caused by occlusion and mastication as well as to static strains during orthodontic tooth movement (5).

During orthodontic treatment, continuous strains of different magnitude and duration are applied. Both fixed and removable appliances can be employed, the strain pattern of which differs significantly. Removable appliances exert intermittent strains to the teeth, whereas fixed appliances produce continuous strains. Animal studies have shown that continuous, as well as intermittent, strains produce the same orthodontic tooth movement (6–8). All types of removable appliances, such as functional, headgears and so on, produce equivalent tooth movement with less than full-time wear (9–10), because even a short exposure to mechanical loading can provoke bone remodelling (11). Fixed appliances exert continuous constant loadings for a significantly extended amount of time, through the use of elastomeric chains, coil springs, and closing loops, before the strain decays either due to strain relaxation of the loading applying mechanism or through tooth movement that decreases the magnitude of deformation of the applying medium.

The total mechanical loading exerted to the teeth by the aforementioned factors is converted into a cellular response (i.e. mechanotransduction) based on a complex network of sensing molecules. Stress fibre formation and focal adhesions organized by Rho family of Ras-related guanosine triphosphatases and activation of several signalling pathways, including the family of mitogen-activated protein kinase (MAPK) cascades (i.e. the ERK, p38, and JNK), activate several specific transcription factors. One of the central transcription factors regarding osteoblastic differentiation is AP-1, which is involved in the regulation of osteoblast-specific genes, including alkaline phosphatase (*ALP*), collagen type I, or osteopontin (*OPN*) (12). AP-1 is a homodimeric/heterodimeric complex consisted of members of the Fos and Jun families of transcription factors, controlling gene expression by binding to specific motifs in the regulatory regions of target genes (13). AP-1 is important for the immediate cellular response to external stimuli (14–15). In this vein, it has already been reported that static mechanical deformation of PDLF upregulates c-Jun and c-Fos (16), upregulates and activates Runx2 via the ERK pathway (17), and increases AP-1 binding in the promoter of ALP (18). Furthermore, cyclic mechanical stimulation in these cells activates ERK, JNK, and p38 MAPK in a RhoK-dependent manner; upregulates *c-fos*; and stimulates the expression of *ALP*, an early marker of osteoblastic differentiation (19–20).

Several studies have already focused on the immediate/early effects of cyclic or static strains on PDLFs. However, the majority of these studies have been performed using a variety of experimental protocols, regarding different strain magnitude, duration and frequency, as well as different culture conditions (5, 20–25). Consequently, different, and in many cases, contradictory data have been reported. Accordingly, the aim of this study was to compare the immediate/early effect of cyclic and static strains of the same magnitude on PDLFs cultured under identical conditions.

Materials and methods

Cells and culture conditions

Human teeth of three healthy normal donors, with age ranging from 9 to 20 years, were extracted in the course of orthodontic treatment after obtaining approval from the Bioethics Committee of the National Centre for Scientific Research 'Demokritos' (No 240/2013-1640). PDL tissue explants were used to develop primary cultures of fibroblasts as previously described (16, 26). In brief, the PDL tissue attached to the apical third of the root surface was scraped off, cut into small pieces, and placed in tissue culture dishes. The fibroblasts (PDLF) released from these tissues were routinely cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml; all from Biochrom AG, Berlin, Germany) and 10 per cent (v/v) fetal bovine serum (FBS), from Gibco BRL (Invitrogen, Paisley, UK) in an environment of 5 per cent carbon dioxide, 85 per cent humidity, and 37°C and were subcultured when confluent by using a trypsin/citrate (0.25 per cent/0.30 per cent, w/v) solution. Early passage cells were used at passages 3–6.

Application of mechanical stretching

Cyclic tensile strain was applied to the cells with a specially designed device, as described previously (20, 27). Briefly, PDLFs were plated onto deformable and optically transparent silicone dishes, which were pre-coated with fibronectin from bovine plasma [Sigma, St Louis, Missouri, USA; 25 ng/ml in 0.5 M sodium chloride (NaCl)–50 mM Tris–hydrochloric acid (HCl), pH = 7.5]. Cells were maintained for 48 h in DMEM supplemented with 10 per cent FBS to adapt to culture conditions, and 24 h prior to mechanical stretching their medium was aspirated and replaced by fresh medium. Cyclic strain (extension 8 per cent, frequency 1 Hz), that is under conditions falling within the range of physiological tissue deformation (28–29), was applied to the cells for the indicated time periods. Static tensile strain (extension 8 per cent) was applied with a novel in-house designed device prepared by Controla (Advanced Technology Equipment, Athens, Greece; Figure 1). In this device, the same silicone dishes were used, and cells were cultured under identical conditions.

Western immunoblot analysis

The western immunoblot analysis was performed as described previously (30). Cells were washed with ice-cold Tris-buffered saline (TBS: 10 mM Tris-HCl, pH = 7.4, 150 mM NaCl) and scraped immediately in hot 2× sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [125 mM Tris-HCl, pH = 6.8, 5 per cent (w/v) SDS, 20 per cent (v/v) glycerol, 125 mM β-mercaptoethanol, and 0.02 per cent (w/v) bromophenol blue] supplemented with protease- and phosphatase-inhibitor cocktails (Sigma). Cell lysates were boiled for 3 minutes, sonicated for 15 seconds, clarified by centrifugation, aliquoted, and stored at −80°C until use. The samples were separated on SDS-PAGE, and the proteins were transferred to PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked with 5 per cent (w/v) non-fat milk in 10 mM Tris-HCl,

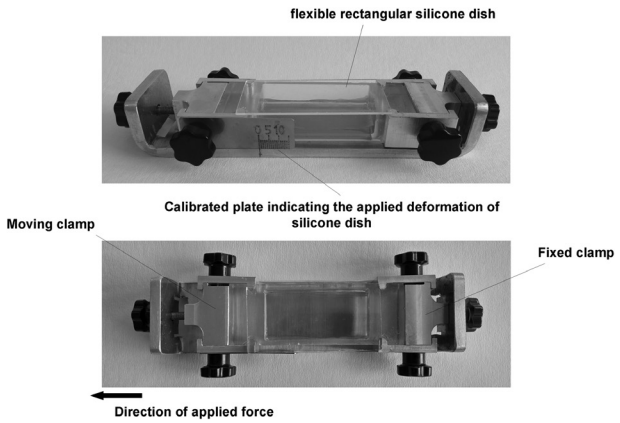

Figure 1. The static stretching apparatus. The rectangular flexible silicone culture dish is held at one end by a fixed clamp and the opposite end by a moving clamp. Strain magnitude can be modified manually by the knob of the moving clamp. A calibrated plate is attached to the moving clamp designating the deformation applied to the flexible silicone dish.

pH = 7.4, 150 mM NaCl, 0.05 per cent Tween-20 (TBS-T) buffer and were incubated overnight at 4°C with the appropriate primary antibodies. Antibodies against p38, phospho-p38 (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185), and SAPK/JNK were purchased from Cell Signaling Technology (Hertfordshire, UK). The antibodies against panERK and phospho-ERK1/2 (Thr202/Tyr204) were from BD Pharmingen (Bedford, MA); the PanActin antibody was from Neomarkers, Lab Vision Corporation (Fremont, California, USA). After washing with 5 per cent non-fat milk, the membranes were incubated with the respective secondary horseradish peroxidase-conjugated antibody (Sigma) for 1.5 h, washed again twice with 5 per cent non-fat milk and once with TBS-T, and finally the immunoreactive bands were visualized on Kodak-X-OMAT AR film by chemiluminescence (ECL kit), according to the manufacturer's instructions (Amersham Biosciences). In all cases, actin was used as a loading control.

Quantitative real-time polymerase chain reaction

Gene expression analysis was performed by quantitative real-time polymerase chain reaction (qRT-PCR), as previously described (30). Total RNA from unstimulated (control) and cyclically- or statically stretched hPDLFs was isolated using Trizol reagent (Life Technologies, Europe, BV). First-strand complementary DNA (cDNA) was synthesized from 500 ng of the total RNA using PrimeScript™ RT Reagent Kit according to the manufacturer's instructions (Takara Bio Inc, Tokyo, Japan). Five microlitres of the cDNA (1:25) per sample was subjected to qRT-PCR using the KAPA SYBR FAST qPCR kit (KAPA Biosystems, Wilmington, Massachusetts, USA). The reaction was carried out in an MX 3000 P QPCR Systems Cycler (Stratagene, La Jolla, California, USA) under the following conditions: denaturation program (95°C for 3 minutes), amplification and quantification program (95°C for 3 seconds, 60–62°C for 20 seconds, 72°C for 10 seconds) repeated 45 times, melting curve program (60–95°C with a heating rate of 0.1°C/s and a continuous fluorescence measurement) and finally a cooling step to 55°C. Data analysis was performed with MxPro QPCR software. Cycle threshold (C_t) values of each target gene were normalized to that of the housekeeping gene GAPDH. The $\Delta\Delta C_t$ method was used to evaluate the relative messenger RNA expression of each gene. The primers used for amplification were *c-fos*: forward 5′-AGA ATC CGA AGG GAA AGG AA-3′, reverse 5′-CTT CTC CTT CAG GTT GG-3′; *c-jun*: forward 5′-CAC GTT AAC AGT GGG TGC CA-3′, reverse 5′-CCC CGA CGG TCT CTC TTC A-3′; and *GAPDH*: forward 5′-GAG TCC ACT GGG GTC TTC-3′, reverse 5′-GCA TTG CTG ATG ATC TTG GG-3′.

Statistical analysis

Hierarchical two-level linear mixed models were used to examine the effect of cell stretching (cyclic versus static) on the expression of *c-fos* and *c-jun* separately, which accounted for the nested nature of the data (observations within donors). Model fit was checked through Kernel density residual plots (data not shown). Natural logarithms of *c-fos* and *c-jun* expression were used in the regression model to allow for normality assumptions. All statistical analyses were conduced with STATA® version 14.1 software (STATA Corporation, College Station, Texas, USA).

Results

Response of early passage PDLFs to cyclic or static mechanical stimulation

Early passage human PDLF (hPDLF), plated on fibronectin-coated silicone dishes, were subjected to cyclic strain (extension 8 per cent, frequency 1Hz) or to static deformation (extension 8 per cent), for various time points. Figure 2 shows that, in contrast to control cells, which were randomly distributed, the cells subjected to cyclic strain for 16 hours tended, to a significant extent, to be aligned perpendicularly to the direction of the strain applied. On the other hand, the cells subjected to static stretching for the same period remained randomly distributed in the culture dish, similar to the control cultures.

Activation of MAPK in response to cyclic and static tensile strain

Activation of MAPK pathways is a major immediate cell response to numerous external stimuli, including mechanical deformation (15, 20). Here, we exposed PDLF to cyclic or static tensile strain for various time points (up to 180 minutes), and the activation of these pathways was evaluated by western blotting analysis. In all subsequent experiments, we compared the responses of cells from the same donor to both types of stress; cells from at least three different donors were used in these experiments, and all gave similar results. As can be seen in Figure 3, cyclic stress activated all three pathways (ERK, p38, and JNK) 15 minutes after stretching application. This activation (i.e. phosphorylation) remained for at least 60 minutes and then returned to basal levels at 180 minutes of stimulation. Interestingly, static tensile strain also activated these pathways 15 minutes after cells' extension. However, this activation lasted at least 30 minutes after stress application and was lost at 60 minutes of stimulation. Quantification of MAPK phosphorylation is presented in Supplementary Table 1.

Regulation of *c-fos* and *c-jun* gene expression by cyclic and static tensile strain

Further downstream of MAPK activation, we studied the effect of the two types of stretching on the expression of the immediate/early genes *c-fos* and *c-jun*, encoding members of the AP-1 transcriptional

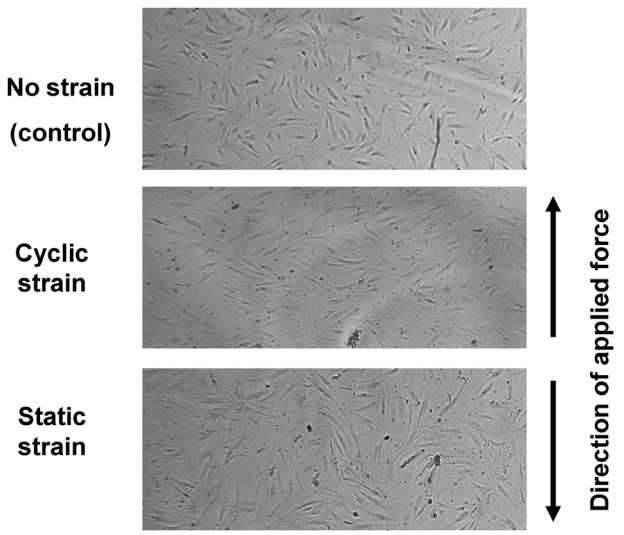

Figure 2. Human PDLFs exhibit discrete orientation in response to long-term cyclic and static tensile stretching. Early passage hPDLFs plated onto fibronectin-coated silicone dishes were subjected either to cyclic (extension 8 per cent, frequency 1 Hz) or to static tensile strain (extension 8 per cent) for 16 hours and were photographed under an inverted microscope. Cyclically stretched cells are aligned nearly perpendicular to the direction of the applied strain (black arrows), whereas cells subjected to static strain, for the same period, remained randomly distributed in the silicone culture dish similarly to their unstimulated counterparts (control). PDLFs = periodontal ligament fibroblasts; hPDLs = human periodontal ligament fibroblasts.

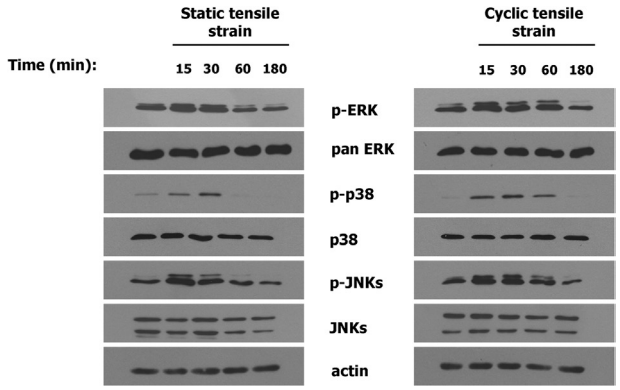

Figure 3. MAPK activation after exposure of hPDLFs to static and cyclic tensile deformation. Early passage hPDLFs, originating from the same donor, plated onto fibronectin-coated silicone dishes were exposed either to static or to cyclic tensile loading. Cell lysates from both statically- and cyclically stretched cells were collected at the indicated time points and the activation of ERK, p38, and JNK/SAPK MAPK kinases was assessed by western blot analysis. Actin was used as a loading control. Representative blots from similar experiments performed in cultures from 3 donors are depicted here. MAPK = mitogen-activated protein kinase; hPDLs = human periodontal ligament fibroblasts.

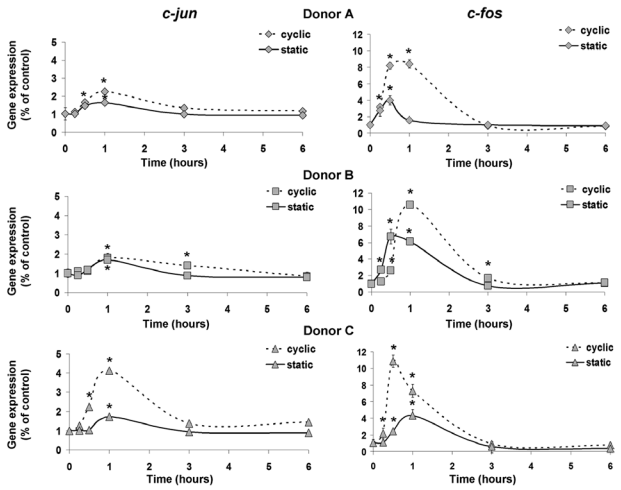

Figure 4. Both static and cyclic mechanical loading activate rapidly the expression of the immediate genes *c-fos* and *c-jun* in hPDLFs. Early passage PDLFs, originating from three different donors (A, B, and C), plated onto fibronectin-coated silicone dishes were subjected either to static or to cyclic tensile stretching for 0.25–6 hours. At the end of the indicated time intervals, messenger RNA was collected and the expression of *c-jun* and *c-fos* was monitored by quantitative real-time polymerase chain reaction. A representative experiment out of three similar ones, performed in triplicates, is presented here. Data are expressed as the mean ± standard deviation. The levels of *c-jun* and *c-fos* mRNA expression were calculated and normalized to the level of GAPDH mRNA. * Student's *t*-test $P < 0.05$: statistically significant differences in comparison with each donor's unstimulated counterparts.

complex, the latter being important for the regulation of expression of osteoblast-specific genes. We found that, in all experiments performed with cells from different donors, both cyclic and static stretching activate *c-fos* as soon as 15 minutes after stress application with the peak of activation at approximately 30 minutes (Figure 4); similarly, *c-jun* increased expression was first found (in most cases) at 30 minutes and the peak was at 60 minutes of stretching (Figure 4). At the peak of the expression, the response to cyclic stretching was significantly higher (as compared to static strain) both for *c-fos* ($P = 0.001$) and for *c-jun* ($P < 0.001$); the expression after both types of stretching returned to the basal levels after 3–6 hours of stimulation. These results indicate that PDLF respond readily to both cyclic and static mechanical deformation, with cyclic strain provoking a more intense response.

Discussion

PDL is continuously exposed to different types of mechanical strains, such as cyclic or static deformation, caused by occlusion and mastication as well as during orthodontic treatment (5). PDL cells and more specifically PDLFs respond to these mechanical loads, transforming them to biochemical signals, for example activation of intracellular signalling pathways and expression of specific genes, eventually leading to PDL repair, remodelling, differentiation, and regeneration. There is an increasing number of studies dealing with the response of PDLFs to cyclic and static mechanical stimulation. Among the most important targets are the activation of the MAPK signalling pathways and the regulation of the *c-fos* and *c-jun* genes, encoding members of the AP-1 transcription factor, the latter playing significant role in the regulation of osteoblast-specific genes, such as *ALP*, collagen I, or *OPN* (16). Different types of mechanical stimulation, of different duration, frequency, and magnitude, have been used in these studies (5). Furthermore, the same type of mechanical deformation has been studied using different experimental setups or devices. For example, the application of mechanical loadings has been studied by cell centrifugation, or alternatively in cells cultured on flexible bottom surfaces extended by vacuum or using uniaxial strain (20, 23, 31). Similarly, static stretching has also been studied under varying conditions of culture and mechanical deformation. Finally, there are no data in the literature comparing the immediate/early responses of these cells to the application of cyclic or static mechanical loading. To this end, we cultured the cells under identical conditions, that is on fibronectin-coated extendable silicone dishes, and tensile stretching has been applied in a well-established device for cyclic deformation (27) and a new home-made device for static extension (Figure 1). First, we studied the activation of the MAPK signalling pathways as they represent a major initial cellular response to a variety of exogenous stresses (14). Previous studies have shown the activation of MAPK in PDLFs in response to a cyclic or static mechanical deformation of varying conditions (type of stress, magnitude, duration, or frequency) (16–18, 20, 32). Here, by using PDLFs cultured under identical conditions, we showed that both cyclic and static stretching of the same magnitude activate all three MAPK pathways (ERK, p38, and JNK). Interestingly, all pathways are rapidly activated with both types of stress, and the only difference found was that the phosphorylation of these kinases was slightly more extended after cyclic stress, as compared to static loading.

The transcription factor AP-1 plays a central role in the regulation of genes that are activated early in osteoblastic differentiation (18, 33–34). In addition, *c-fos* upregulation is considered as a classical response to mechanical stress. Previous studies have shown in PDLFs the upregulation of the c-Fos and c-Jun, members of the AP-1 complex, in response to mechanical stimulation, as well as the potentiation of AP-1 binding in the promoter of the *ALP* gene, a marker of osteoblastic differentiation (16, 18, 20, 32). Notably, it has been shown that members of the MAPK signalling pathways control the expression of AP-1 components after mechanical stimulation (16, 18, 32). The comparative study performed here has shown that both static and cyclic stretching upregulate the *c-fos* and the *c-jun* genes.

The peak of activation was observed 30 minutes for *c-fos* and 60 minutes for *c-jun* after stimulation, while the expression returned to the basal levels after 3–6 hours. However, in all cases, cyclic stretching provokes significantly higher induction of both genes in cells from three different donors, in agreement with the more extended MAPK activation. In this vein, we have shown that a long-term (i.e. 16 hours) cyclic tensile stretching provokes an alignment of PDLFs in a direction, approximately perpendicular to the stretch direction, whereas signs of alignment can be observed from 6 hours of mechanical strain. This is in agreement with our published data on PDLFs (20), as well as with previous results on skin fibroblasts and osteoblasts (27), suggesting an avoidance response to stretching application (35). In contrast, after the exposure of PDLFs to static tensile stretching of the same duration and magnitude, the cells remain in a random orientation in culture, similarly to control (unstretched) cultures. This does not represent a return to the basal condition, as even short duration of static stretching (i.e. 1–6 hours) does not provoke any changes in cell orientation (not shown here).

Conclusions

In summary, the results of the present study indicate that under identical culture conditions PDLFs, originating from the same donors, have similar, although not identical, immediate/early responses when exposed to cyclic or static tensile stretching of the same magnitude, with cyclic strain provoking more prolonged MAPK activation and more intense *c-jun* and *c-fos* gene expression. In line with the above, only cells exposed to cyclic stress for longer periods are distributed approximately perpendicular to the axis of the applied strain, whereas cells exposed to static loading remain in a random orientation in culture.

Supplementary material

Supplementary material is available at *European Journal of Orthodontics* online.

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Conflict of interest

None declared.

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