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Original Article

Effect of Stretching Stress on Gene Transcription Related to Early-phase Differentiation in Rat Periodontal Ligament Cells

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Abstract

Mechanical stress such as occlusal and orthodontic loading has been suggested to induce a homeostatic and regenerative response in periodontal ligament (PDL), but the underlying mechanism remains to be clarified. The purpose of this study was to investigate expression of mRNAs encoding proteins involved in osteogenesis and homeostasis by PDL cells following application of tensile stress and characterize the relationship between such expression and the regenerative and homeostatic functions of the PDL. PDL cells were obtained from rats and stretched by 9% or 18% at a frequency of 6 cycles/min for 12 hr to 5 days in a FX-4000T™ culture system. After stretching, expression of mRNAs encoding collagen type I (Col-I), alkaline phosphatase (ALP), bone morphogenetic protein-2 (BMP-2), bone morphogenetic protein-4 (BMP-4), heat shock protein 70 (HSP70) and basic fibroblast growth factor (bFGF) was investigated. The highest levels of Col-I, ALP and BMP-2 mRNA expression occurred at 12 hr, while those of BMP-4 and HSP70 occurred at 1 day and 5 days, respectively. Expression levels of Col-I, ALP, BMP-2, BMP-4 and HSP70 increased magnitude-dependently with stretching force in the

stretching groups. In contrast, expression of bFGF mRNA showed statistically significant reduction in both stretching groups, with the largest reduction seen in the 9% stretching group (p<0.01). These results suggest that stretching of PDL cells provokes significant increases in expression of factors promoting osteogenic differentiation and HSP70, which protects PDL cells undergoing mechanical stress and contributes to maintenance of PDL homeostasis. However, expression of bFGF was restrained. Reduced expression of bFGF mRNA suggested that there was an optimum magnitude of stretching force for increasing expression.

Key words:　　Periodontal ligament—Mechanical stress—Homeostasis—Regeneration

Introduction

Orthodontic tooth movement, periodontal treatment and tooth transplantation and replantation induce a homeostatic and regenerative response in the periodontal ligament (PDL). In particular, it has been suggested that the PDL acts as a buffer against various mechanical stimuli and prevents ankylosis following tooth movement, transplantation and replantation due to suppression of differentiation of PDL cells to osteoblasts and/or cementoblasts by occlusal forces, thus maintaining the fixed spacing of the teeth[15,18]. However, exactly how mechanical stress due to occlusal forces induces a homeostatic and regenerative response in the PDL remains to be clarified.

Using the Flexercell® Tension Plus™ Unit (Flexercell), earlier studies have investigated the effect of mechanical stress on the secretion of transforming growth factor-beta 1 (TGF-β1) and macrophage colony stimulating factor (M-CSF)[6], and the role of epidermal growth factor (EGF) and its receptor (EGF-R) in proliferation and differentiation of PDL cells under mechanical stress-loaded conditions *in vitro*[9]. Furthermore, the influence of cyclic mechanical stress of varying magnitude on changes in mRNA expression levels of collagen type I (Col-I), decorin genes and alkalin phosphatase (ALP) activity[12], the effect of mechanical tension force on interleukin-1β (IL-1β) synthesis and bone-resorbing activity in human PDL cells[17] and the effect of different magnitudes of tension force have been reported on prostaglandin E_2 (PGE_2)

production and inositol trisphosphate (IP_3) levels and plasminogen activator (PA) activity in PDL cells *in vitro*[23,24]. These investigations have suggested that mechanical stress applied using a Flexercell® can induce different responses and regulate differentiation of PDL cells[6,9,12,17,23,24].

Nevertheless, how mechanical stress induces regeneration and homeostatic maintenance of the PDL remains to be fully clarified. The purpose of this study was to investigate expression of mRNAs encoding proteins involved in osteogenesis and homeostasis by PDL cells following application of tensile stress and characterize the relationship between such expression and the regenerative and homeostatic functions of the PDL. We investigated expression of mRNAs encoding Col-I, ALP, bone morphogenetic protein-2 (BMP-2) and bone morphogenetic protein-4 (BMP-4) as indicators of osteogenesis, basic fibroblast growth factor (bFGF) as an indicator of proliferative fibroblasts and heat shock protein 70 (HSP70) as an indicator of protective response in the PDL.

Materials and Methods

1. Isolation of PDL cells

All experiments were performed in accordance with the Guidelines for Use of Experimental Laboratory Animals at the Animal Facility of Tokyo Dental College. Four-week-old male Sprague-Dawley rats were anesthetized with an intraperitoneal injection of 2.5% sodium thiopental (100 mg/kg Ravonal®,

Mitsubishi Tanabe Pharma Corp., Osaka, Japan). The mandibular and maxillary incisors were then detached with an improved elevator and extracted mechanically. The apical end of each incisor containing the tooth germ and the labial side containing soft tissue other than PDL or pulp tissue were removed mechanically using a surgical blade. The incisors were then bisected axially and placed in culture medium containing a 10-times concentration of antibiotics for 30 min at room temperature to prevent infection. The bisected incisors were then placed with the PDL side down in 35-mm culture dishes and incubated to allow initial adhesion of cells. Tissues were soaked and cultured in medium containing α-minimum essential medium (α-MEM) (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), 100 IU/ml penicillin (Invitrogen Corp.), 100 μg/ml streptomycin (Invitrogen Corp.), 50 μg/ml gentamicin (Invitrogen Corp.) and 0.3 μg/ml amphotericin B (Invitrogen Corp.) and kept in a humidified incubator in the presence of 95% air and 5% CO_2 at 37°C. When cells reached confluence, they were detached with trypsin-EDTA (Invitrogen Corp., 0.05% trypsin, 0.53 mM EDTA·4Na) in PBS for 5 min at 37°C and subcultured in culture dishes. Culture media were changed every 48–72 hr. Cells were passaged three times for use in the experiments.

2. Cell culture treatment

PDL cells were seeded (1×10^5 cells/well) onto BioFlex® plates (FLEXCELL Int. Corp., McKeesport, PA, USA) and cultured for 3 days until confluent. The medium was then replaced with the same medium described above, except that it contained 2% FBS instead of 15%. The cells were cultured for a further 1 day, followed by another change of the same medium containing 2% FBS, and then used for the experiments. Cells in the stretching groups were placed in the BioFlex® Loading Stations™ of the FX-4000T™ Flexercell® Tension Plus™ Unit (FLEXCELL Int. Corp.) and subjected to 9% or 18% stretching loads at a frequency 6 cycles/min for 12 hr, 1, 3 or 5 days. Cells cultured under the same conditions but without application of any load were used as the control group.

3. Quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR)

1) Total RNA extraction

Total RNAs were extracted from cells in each group using the acid guanidium thiocyanate-phenol-chloroform method (TRIzol reagent, Invitrogen Corp.) according to the manufacturer's instructions. Total RNA pellets were dissolved in RNase-free water and total RNA concentrations measured by absorbance using the Nanodrop® (ND-1000 Spectrophotometer: Laboratory & Medical Supplies Co., Ltd., Tokyo, Japan). Total RNAs were reverse transcribed into complementary DNAs (cDNA) using the Takara RNA PCR Kit (Takara, Tokyo, Japan) and stored at $-80°C$ until analyzed.

2) QRT-PCR assays

QRT-PCR assays were performed with the LightCycler™ using double-stranded DNA dye SYBER Green I (Roche Diagnostics GmbH, Manheim, Germany). The PCR conditions and primer sequences used for rat Col-I, ALP, BMP-2, BMP-4, bFGF, HSP70 and β-actin (as internal control) are shown in Table 1. Quantification was performed by comparing levels obtained with standardized sample and ratios of Col-I, ALP, BMP-2, BMP-4, bFGF and HSP70 mRNAs were normalized against β-actin. In this experiment, we compared expression of β-actin with that of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). As the value for β-actin was stable, we decided to use β-actin.

Results

1. QRT-PCR assays

A maximal and significant increase in expression of Col-I mRNA was observed at 12 hr, which then decreased from 1 day onwards. Expression of Col-I in the 9% stretching group was approximately 1.2-fold that in the control group (control group: 12 hr: 1.8 ± 0.63, 1 d: 1.3 ± 0.72, 3 d: 1.1 ± 0.69,

Table 1   Primers used for quantitative real-time polymerase chain reaction

		Primer sequences	PCR conditions	Product sizes
Col-I	forward:	5′-CAAGACAGTCATCGAATACA-3′	30 cycles	252 bp
	reverse:	5′-AGTCCATGTGAAATTGTCTC-3′	(95°C 10 sec, 60°C 5 sec, 72°C 12 sec)	
ALP	forward:	5′-GGCTCTCTCCAAGACGTACAA-3′	35 cycles	230 bp
	reverse:	5′-GCGTGGTTCACCCGAGTGG-3′	(95°C 10 sec, 60°C 5 sec, 72°C 12 sec)	
BMP-2	forward:	5′-CCCTATATGCTCGACCTGT-3′	35 cycles	137 bp
	reverse:	5′-GAAAGTTCCTCGATGGCT-3′	(95°C 10 sec, 60°C 5 sec, 72°C 12 sec)	
BMP-4	forward:	5′-CCGGATTACATGAGGGAAT-3′	30 cycles	208 bp
	reverse:	5′-GGATGCTGCTGAGGTTAAAG-3′	(95°C 10 sec, 60°C 5 sec, 72°C 12 sec)	
bFGF	forward:	5′-GGCTATGAAGGAAGATGGAC-3′	35 cycles	141 bp
	reverse:	5′-GTTCGTTTCAGTGCCACA-3′	(95°C 10 sec, 60°C 5 sec, 72°C 12 sec)	
HSP70	forward:	5′-GTGTGCAACCCGATCATCAG-3′	35 cycles	180 bp
	reverse:	5′-CACCAGCAGCCATCAAGAGT-3′	(95°C 10 sec, 60°C 5 sec, 72°C 12 sec)	
β-actin	forward:	5′-CCTGTATGCCTCTGGTCGTA-3′	30 cycles	260 bp
	reverse:	5′-CCATCTCTTGCTCGAAGTCT-3′	(95°C 10 sec, 60°C 5 sec, 72°C 12 sec)	

Collagen type I: Col-I, Alkaline phosphatase: ALP, Bone morphogenetic protein-2: BMP-2,
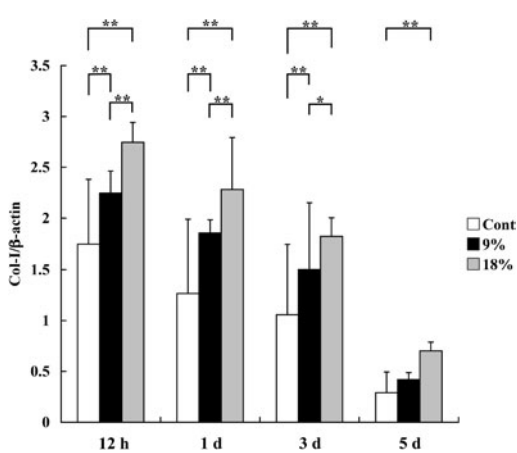Bone morphogenetic protein-4: BMP-4, Basic fibroblast growth factor: bFGF, Heat shock protein70: HSP70

Fig. 1   Expression of Col-I mRNA determined by quantitative real-time RT-PCR in rat PDL cells subjected to 9% or 18% stretching loads at frequency of 6 cycles/min for 12 hr, 1, 3 or 5 days. A maximal and significant increase in expression of Col-I mRNA was observed at 12 hr, which then decreased from 1 day onwards. Data are the mean ± standard deviation of triplicate assays, and were analyzed using a two-way analysis of variance. The values were then compared using the Fisher's protected least significant difference test (**p<0.01, *p<0.05).
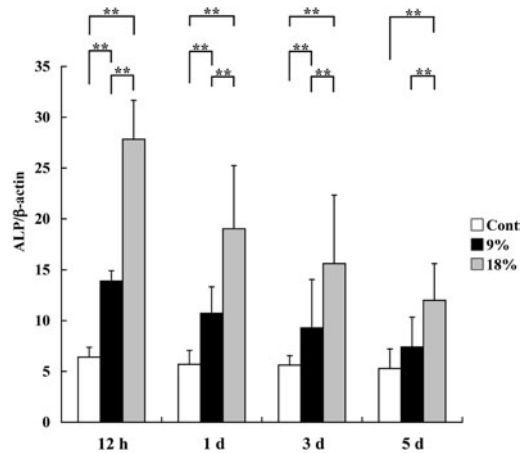

Fig. 2   Expression of ALP mRNA in rat PDL cells subjected to 9% or 18% stretching loads at frequency of 6 cycles/min for 12 hr, 1, 3 or 5 days. A maximal and significant increase in expression of ALP mRNA was observed at 12 hr, which then decreased from 1 day onwards. Data are the mean ± standard deviation of triplicate assays, and were analyzed using a two-way analysis of variance. The values were then compared using the Fisher's protected least significant difference test (**p<0.01).

5 d: 0.3 ± 0.20, respectively) at 12 hr, but declined from 1 day onwards (12 hr: 2.2 ± 0.22, 1 d: 1.8 ± 0.13, 3 d: 1.5 ± 0.65, 5 d: 0.4 ± 0.07, respectively). Expression of Col-I in the 18% stretching group was approxi-

mately 1.5 and 1.3-fold that in the control group at 12 hr and 1 day, respectively, but decreased at 3 and 5 days (12 hr: 2.7 ± 0.19, 1 d: 2.3 ± 0.51, 3 d: 1.8 ± 0.18, 5 d: 0.7 ± 0.09, respectively) (Fig. 1).
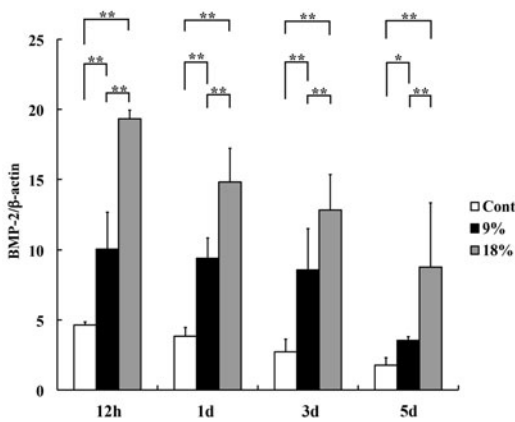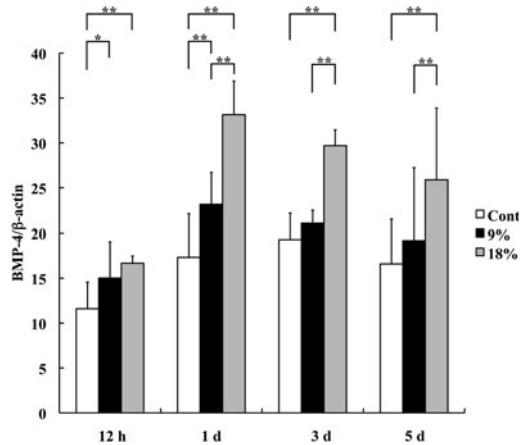
Fig. 3 Expression of BMP-2 mRNA in rat PDL cells subjected to 9% or 18% stretching loads at frequency of 6 cycles/min for 12 hr, 1, 3 or 5 days. Expression of BMP-2 mRNA reached maximum at 12 hr, but began to decrease at 1 day. Data are the mean ± standard deviation of triplicate assays, and were analyzed using a two-way analysis of variance. The values were then compared using the Fisher's protected least significant difference test (**p<0.01, *p<0.05).

Fig. 4 Expression of BMP-4 mRNA in rat PDL cells subjected to 9% or 18% stretching loads at frequency of 6 cycles/min for 12 hr, 1, 3 or 5 days. A maximal and significant increase in expression of BMP-4 was observed at 1 day, which then decreased from 3 days onwards. Data are the mean ± standard deviation of triplicate assays, and were analyzed using a two-way analysis of variance. The values were then compared using the Fisher's protected least significant difference test (**p<0.01, *p<0.05).

A maximal and significant increase in expression of ALP mRNA was observed at 12 hr, which then decreased from 1 day onwards. Expression of ALP in the 9% stretching group (12 hr: 13.9 ± 1.02, 1 d: 10.7 ± 2.61, 3 d: 9.3 ± 4.74, 5 d: 7.4 ± 2.93, respectively) was 2.2-, 1.9-, 1.7- and 1.4-fold that in the control group (12 hr: 6.4 ± 0.97, 1 d: 5.7 ± 1.37, 3 d: 5.6 ± 0.92, 5 d: 5.3 ± 1.93, respectively) at 12 hr, 1, 3 and 5 days, respectively. Expression of ALP in the 18% stretching group (12 hr: 27.8 ± 3.83, 1 d: 19.0 ± 6.20, 3 d: 15.6 ± 6.75, 5 d: 12.0 ± 3.60, respectively) was 4.3-, 3.3-, 2.8- and 2.2-fold that in the control group at 12 hr, 1, 3 and 5 days, respectively (Fig. 2).

Expression of BMP-2 mRNA reached a maximum at 12 hr, but began to decrease at 1 day. Expression of BMP-2 in the 9% stretching group (12 hr: 10.1 ± 2.59, 1 d: 9.4 ± 1.45, 3 d: 8.6 ± 2.95, 5 d: 3.5 ± 0.27, respectively) was approximately 2.2-, 2.5-, 3.2- and 1.9-fold that in the control group (12 hr: 4.6 ± 0.22, 1 d: 3.8 ± 0.62, 3 d: 2.7 ± 0.90, 5 d: 1.8 ± 0.54, respectively) at 12 hr, 1, 3 and 5 days, respectively. Expression of BMP-2 mRNA in the 18% stretching group showed an approximately 4.2-, 3.9-, 4.7- and 4.9-fold increase in comparison with that in the control group (12 hr: 19.3 ± 0.61, 1 d: 14.8 ± 2.41, 3 d: 12.8 ± 2.53, 5 d: 8.8 ± 4.54, respectively) at 12 hr, 1, 3 and 5 days, respectively (Fig. 3).

Maximal expression of BMP-4 mRNA occurred at 1 day, and then began to decrease at 3 days. Expression of BMP-4 in the 9% stretching group (12 hr: 15.0 ± 4.02, 1 d: 23.2 ± 3.56, 3 d: 21.1 ± 1.45, 5 d: 19.2 ± 8.07, respectively) was approximately 1.3-, 1.3-, 1.0- and 1.2-fold that in the control group (12 hr: 11.6 ± 2.97, 1 d: 17.3 ± 4.80, 3 d: 19.3 ± 2.91, 5 d: 16.6 ± 4.93, respectively) at each experimental period, respectively. Expression of BMP-4 in the 18% stretching group (12 hr: 16.7 ± 0.79, 1 d: 33.1 ± 3.74, 3 d: 29.7 ± 1.76, 5 d: 25.9 ± 7.98, respectively) was approximately 1.4-, 1.9-, 1.5- and 1.6-fold that in the control group at each experimental period, respectively (Fig. 4).

In contrast, expression of bFGF mRNA in the 18% stretching group was the same as that in the control group (12 hr: 2.7 ± 0.48, 1 d:
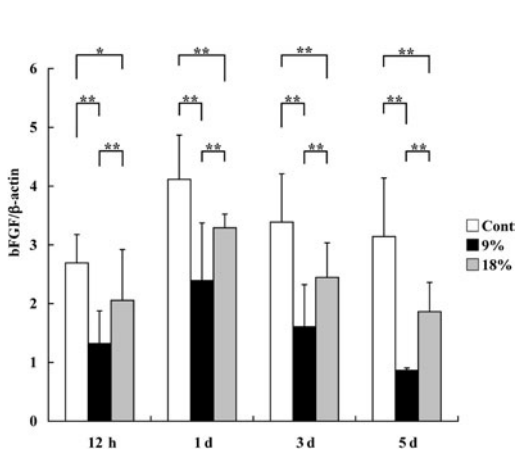
Fig. 5 Expression of bFGF mRNA in rat PDL cells subjected to 9% or 18% stretching loads at frequency of 6 cycles/min for 12 hr, 1, 3 or 5 days. Expression of bFGF mRNA in 18% stretching group was the same as that in control group at 1 day, but was lower at all other experimental periods in both 9% and 18% stretching groups. Data are the mean ± standard deviation of triplicate assays, and were analyzed using a two-way analysis of variance. The values were then compared using the Fisher's protected least significant difference test (**p<0.01, *p<0.05).
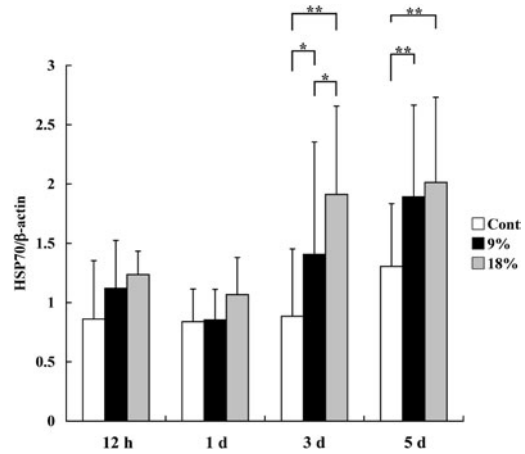
Fig. 6 Expression of HSP70 mRNA in rat PDL cells subjected to 9% or 18% stretching loads at frequency of 6 cycles/min for 12 hr, 1, 3 or 5 days. A maximal and significant increase in expression of HSP70 was observed at 5 days. Data are the mean ± standard deviation of triplicate assays, and were analyzed using a two-way analysis of variance. The values were then compared using the Fisher's protected least significant difference test (**p<0.01, *p<0.05).

4.1 ± 0.76, 3 d: 3.4 ± 0.82, 5 d: 3.1 ± 0.99, respectively) at 1 day, but was reduced at all other experimental periods in both the 9% and 18% stretching groups. Expression of bFGF in the 18% stretching group (12 hr: 2.1 ± 0.87, 1 d: 3.3 ± 0.23, 3 d: 2.4 ± 0.59, 5 d: 1.9 ± 0.50, respectively) was higher than that in the 9% group (12 hr: 1.3 ± 0.56, 1 d: 2.4 ± 0.98, 3 d: 1.6 ± 0.71, 5 d: 0.9 ± 0.04, respectively) at each experimental period (Fig. 5).

Maximal expression of HSP70 mRNA occurred at 5 days. Expression of HSP70 in the 9% stretching group (12 hr: 1.1 ± 0.40, 1 d: 0.9 ± 0.26, 3 d: 1.4 ± 0.95, 5 d: 1.9 ± 0.77, respectively) was approximately 1.6- and 1.5-fold that in the control group (12 hr: 0.9 ± 0.49, 1 d: 0.8 ± 0.27, 3 d: 0.9 ± 0.57, 5 d: 1.3 ± 0.53, respectively) at 3 and 5 days, respectively, although it was the same level as that in the control group at 12 hr to 3 days. Expression of HSP70 in the 18% stretching group (12 hr: 1.2 ± 0.20, 1 d: 1.1 ± 0.31, 3 d: 1.9 ± 0.75, 5 d: 2.0 ± 0.72, respectively) was approximately 2.1- and 1.5-fold that in the

control group at 3 and 5 days, and was higher than that in the control group at all other experimental periods (Fig. 6). No morphological differences were observed between the experimental and control groups, which consisted of fibroblasts and epithelium-like cells (data not shown).

Discussion

The homeostatic and regenerative response of the PDL to mechanical stress is the most significant with respect to periodontal therapy, transplantation and/or replantation of teeth and orthodontic tooth movement[15]. A mechanism has been hypothesized to suppress synthesis of bone and cementum in order to maintain a fixed space in the PDL[18]. However, how occlusal pressure induces a homeostatic and regenerative response in the PDL remains to be clarified.

In this study, we assumed that Col-I, ALP, BMP-2 and BMP-4 contributed to osteo-

genesis[3–5,7–9,12–14,17–19,21-25)], that HSP70 was involved in homeostasis[1,16)], and that bFGF was closely involved in both functions[11,18,20)].

Although the magnitude of human bite force varies, 500–3,000 g was selected as the standard force for clinical studies[2)]. A previous *in vitro* study of PDL cells using a Flexercell® demonstrated that application of 500 gf orthodontic force to human upper incisors corresponded to about 23% stretching of the PDL on the tension side[10)], and an 18% stretching rate corresponded to 400 gf of orthodontic force[11)]. Most other investigations of the PDL employing a Flexercell employed a stretching rate of 18% as the upper limit at a frequency of 6 cycles/min[9,12,17,24)]. Based on these earlier results, we selected a 9% stretching rate as a light load and an 18% stretching rate as a heavy load.

Our results showed that expression of Col-I mRNA increased with an increase in stretching force. This supports the studies of Howard *et al.* and He *et al.* and suggests that stretching stress facilitates cellular differentiation of PDL cells into osteoprogenitor cells[3,5,19)]. It has been demonstrated that mechanical stretching stress may modulate the increase magnitude-dependently of Col-I synthesis in PDL cells. Expression of ALP, a marker for early osteogenic differentiation, is reduced when extracellular matrix maturation is initiated[19,25)]. Contradictory results on ALP activity in PDL cells have been reported in previous studies using a Flexercell[9,12,25)]. Some studies suggested that PE_2 and IL-1β induced by a cyclic stretching force mediated a decrease in ALP activity in PDL cells[12,17,21,23)]. Other studies, however, have indicated that an increase in ALP activity is a sign of osteoblastic differentiation in PDL cells[7,13)]. Our results showing that expression of ALP increased remarkably in both the 9% and 18% stretching groups suggest that application of a stretching load induces osteoblastic differentiation in PDL cells. BMPs, multifunctional growth factors belonging to the transforming growth factor-beta super-family, participate in the differentiation of undifferentiated mesenchymal cells to osteoblasts[14)]. It has been reported that BMP-2 enhances expression of markers for osteoblastic differentiation such as ALP[4,7)], and also promotes osteoblastic differentiation of human PDL cells to decrease cell proliferation[8)]. Increased expression of ALP mRNA in this study also suggests that cyclic stretching force affects PDL metabolism magnitude-dependently. BMP-4 increased mRNA levels of BMP-2 and ALP[22)], and the expression pattern was different from that in our results. Accordingly, our results showing increased mRNA expression of Col-I, ALP, BMP-2 and BMP-4 suggest that PDL cells differentiate into osteoblasts due to stretching force.

Previous *in vitro* and *in vivo* studies applying recombinant bFGF to PDL indicated that bFGF induced 1) remarkable cellular proliferation, 2) decreased of Col-I mRNA, 3) inhibition of both ALPase induction and calcification, and 4) new formation of PDL with cementum and bone. From these results, it is suggested that bFGF participates in the suppression of PDL cells for osteogenic differentiation and the promotion of the regeneration process by vasculogenesis and cellular proliferation[11,20)]. Taking the results of these studies together with our present results, we surmised that PDL cells subjected to mechanical stretching by Flexercell secreted bFGF, which caused the PDL cells to suppress osteogenic differentiation and enhance proliferation of undifferentiated cells. Consequently, it is possible that mechanical stretching is related to regeneration and homeostasis in PDL.

HSP70 is known to be a protective factor for cells against adverse effects of stress and a molecular chaperone to regulate protein folding and assembly, and HSP70 levels are increased under mechanical stress[1)]. A study on orthodontic tooth movement in rat demonstrated that mRNA expression of HSP70 in pulp cells was higher in the experimental group than in the control group at all time points. These results suggest that orthodontic tooth movement causes degenerative changes and apoptosis in pulp cells, while pulp homeostasis is maintained at the genetic level[16)]. Our results showing increased expression of HSP70 mRNA acting on inhibition of apoptosis sug-

gest that HSP70 protects PDL cells undergoing mechanical stress and contributes to maintenance of PDL homeostasis.

In conclusion, the remarkable increase in expression of osteogenic indicators in PDL cells observed in this study suggests that application of a stretching force induces osteogenic differentiation. We concur with the hypothesis that bFGF suppresses induction of ALP activity and mineralization and promotes proliferation of human PDL cells[11,20]. In this study, expression of bFGF was low at all experimental periods in both stretching groups, which indicates that a stretching stimulus reduces bFGF expression in PDL cells. The results of this study indicate that a stretching force reduces bFGF expression in PDL cells, thus decreasing cellular proliferation while increasing ALP activity and osteogenic differentiation.

Acknowledgements

References

1) Becker J, Craig EA (1994) Heat-shock proteins as molecular chaperones. Eur J Biochem 219: 11–23.

2) Coffey JP, Williams WN, Turner GE, Mahan PE, Lapointe LL, Cornell CE (1989) Human bite force discrimination using specific maxillary and mandibular teeth. J Oral Rehabil 16:529–536.

3) He Y, Macarak EJ, Korostoff JM, Howard PS (2004) Compression and tension: differential effects on matrix accumulation by periodontal ligament fibroblasts *in vitro*. Connect Tissue Res 45:29–39.

4) Hou LT, Li TI, Liu CM, Liu BY, Liu CL, Mi HW (2007) Modulation of osteogenic potential by recombinant human bone morphogenic protein-2 in human periodontal ligament cells: effect of serum, culture medium, and osteoinductive medium. J Periodontal Res 42:244–252.

5) Howard PS, Kucich U, Taliwal R, Korostoff JM (1998) Mechanical forces alter extracellular matrix synthesis by human periodontal ligament fibroblasts. J Periodontal Res 33:500–508.

6) Kimoto S, Matsuzawa M, Matsubara S, Komatsu T, Uchimura N, Kawase T, Saito S (1999) Cytokine secretion of periodontal ligament fibroblasts derived from human deciduous teeth: effect of mechanical stress on the secretion of transforming growth factor-beta 1 and macrophage colony stimulating factor. J Periodontal Res 34:235–243.

7) Kobayashi M, Takiguchi T, Suzuki R, Yamaguchi A, Deguchi K, Shionome M, Miyazawa Y, Nishihara T, Nagumo M, Hasegawa K (1999) Recombinant human bone morphogenetic protein-2 stimulates osteoblastic differentiation in cells isolated from human periodontal ligament. J Dent Res 78:1624–1633.

8) Markopoulou CE, Vrotsos IA, Vavouraki HN, Dereka XE, Mantzavinos ZS (2003) Human periodontal ligament cell responses to recombinant human bone morphogenetic protein-2 with and without bone allografts. J Periodontol 74:982–989.

9) Matsuda N, Yokoyama K, Takeshita S, Watanabe M (1998) Role of epidermal growth factor and its receptor in mechanical stress-induced differentiation of human periodontal ligament cells *in vitro*. Arch Oral Biol 43:987–997.

10) Mühkeman HR, Zander HA (1954) Tooth mobility (III) The mechanism of tooth mobility. J Periodontol 25:128–137.

11) Murakami S, Takayama S, Ikezawa K, Shimabukuro Y, Kitamura M, Nozaki T, Terashima A, Asano T, Okada H (1999) Regeneration of periodontal tissues by basic fibroblast growth factor. J Periodontal Res 34:425–430.

12) Ozaki S, Kaneko S, Podyma-Inoue KA, Yanagishita M, Soma K (2005) Modulation of extracellular matrix synthesis and alkaline phosphatase activity of periodontal ligament cells by mechanical stress. J Periodontal Res 40:110–117.

13) Ozawa H, Imamura K, Abe E, Takahashi N, Hiraide T, Shibasaki Y, Fukuhara T, Suda T (1990) Effect of a continuously applied compressive pressure on mouse osteoblast-like cells (MC3T3-E1) *in vitro*. J Cell Physiol 142: 177–185.

14) Sato M, Ochi T, Nakase T, Hirota S, Kitamura Y, Nomura S, Yasui N (1999) Mechanical

tension-stress induces expression of bone morphogenetic protein (BMP)-2 and BMP-4, but not BMP-6, BMP-7, and GDF-5 mRNA, during distraction osteogenesis. J Bone Miner Res 14:1084–1095.

15) Schroeder HE (1986) The Periodontium, Handbook of Microscopic Anatomy, Oksche A, Vollrath L, volume V/5 eds., pp. 182–233, Springer-Verlag, Berlin.

16) Shigehara S, Matsuzaka K, Inoue T (2006) Morphohistological change and expression of HSP70, osteopontin and osteocalcin mRNAs in rat dental pulp cells with orthodontic tooth movement. Bull Tokyo Dent Coll 47:117–124.

17) Shimizu N, Yamaguchi M, Goseki T, Ozawa Y, Saito K, Takiguchi H, Iwasawa T, Abiko Y (1994) Cyclic-tension force stimulate interleukin-1 beta production by human periodontal ligament cells. J Periodontal Res 29:328–333.

18) Shimono M, Ishikawa T, Ishikawa H, Matsuzaki H, Hashimoto S, Muramatsu T, Shima K, Matsuzaka K, Inoue T (2003) Regulatory mechanisms of periodontal regeneration. Microsc Res Tech 60:491–502.

19) Stein GS, Lian JB, Owen TA (1990) Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB J 4:3111–3123.

20) Takayama S, Murakami S, Miki Y, Ikezawa K, Tasaka S, Terashima A, Asano T, Okada H (1997) Effects of basic fibroblast growth factor on human periodontal ligament cells. J Periodontal Res 32:667–675.

21) Takeshita A, Niu ZG, Hanazawa S, Takara I, Higuchi H, Katayama I, Kitano S (1992) Effect of interleukin-1 beta on gene expressions and functions of fibroblastic cells delived from human periodontal ligament. J Periodontal Res 27:250–255.

22) Xu WP, Shiba H, Mizuno N, Uchida Y, Mouri Y, Kawaguchi H, Kurihara H (2004) Effect of bone morphogenetic proteins-4, -5 and -6 on DNA synthesis and expression of bone-related proteins in cultured human periodontal ligament cells. Cell Biol Int 28:675–682.

23) Yamaguchi M, Shimizu N, Goseki T, Shibata Y, Takiguchi H, Iwasawa T, Abiko Y (1994) Effect of different magnitudes of tension force on prostaglandin E2 production by human periodontal ligament cells. Arch Oral Biol 39:877–884.

24) Yamaguchi M, Shimizu N, Ozawa Y, Saito K, Miura S, Takiguchi H, Iwasawa T, Abiko Y (1997) Effects of tension-force on plasminogen activator activity from human periodontal ligament cells. J Periodontal Res 32:308–314.

25) Yamaguchi M, Shimizu N, Shibata Y, Abiko Y (1996) Effects of different magnitudes of tension-force on alkaline phosphatase activity in periodontal ligament cells. J Dent Res 75:889–894.

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