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Static tensional forces increase osteogenic gene expression in three-dimensional periodontal ligament cell culture

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Orthodontic tooth movement results from the combinational process of both bone resorption and formation in the compressive and tension sides, respectively. However, the genes responsible for new bone formation in tension sides have not been determined. In this study, we used DNA microarray and real-time RT-PCR to identify genes in human periodontal ligament (PDL) cells that undergo significant changes in expression in response to static tensional forces (2 or 12 hours). The genes found were *alkaline phospatase (ALP), matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF),* and several collagen genes. Furthermore, an ELISA evaluating the expression of VEGF, type IV collagen and MMP-2 found levels significantly increased after 24 and 72 hours ($P < 0.05$). ALP activity was also increased after 24 hours ($P < 0.05$). Collectively, we found the genes up-regulated in our study by the static tensional force are related to osteogenic processes such as matrix synthesis and angiogenesis. [BMB reports 2009; 42(7): 427-432]

INTRODUCTION

During orthodontic tooth movement the mechanical stress applied to the tooth is transmitted to the alveolar bone by the periodontal ligament (PDL). Indeed, dynamic processes mediated by the PDL are responsible for remodeling the alveolar bone and providing mechanical stresses in the edentulous alveolar ridge necessary to prevent severe bone resorption (1). Therefore, the presence of PDL is highly important in maintaining the physiology of periodontal tissue such that if damaged by trauma, the tooth will be ankylosed and the patient may have growth problems in the alveolar bone (2). As dental implants lack PDL, the alveolar bone around the dental implant has limited advantages compared to normal periodontal tissue (3).

Mechanical stresses applied to PDL may either be in the form of compression, in which bone resorption is observed, and/or tension, in which a new alveolar bone is formed (4). These responses to mechanical stress have been used for orthodontic tooth movement and both are dependent on the intensity and duration of the applied force (4). Recently, the molecular mechanism of bony resorption in the compressive side was investigated by DNA microarray technology and a three-dimensional cell culture system (5, 6). There it was shown the expression of interleukin-6 (IL-6), a strong osteoclastogenesis-activating cytokine, increased in response to a static compressive force in PDL cells (5, 6). Moreover, the activity of alkaline phosphatase (ALP) in PDL cells decreased by the same static compressive force (5). In contrast, tension applied to the tooth causes newly alveolar bone to form in the tension side (4). Indeed, 12% uni-axial cyclic tension increases the expression of ALP (7). However, other studies show that cyclic tension decreases ALP activity (8). The conflicting results may be due to differences in the experimental system. Considering that ALP activity is an indicator of new bone formation, ALP activity is expected to increase when tension is applied. To replicate *in vivo* conditions, we used the three-dimensional collagen culture system for application of mechanical force. Such a system is much more similar to physiological conditions than the one-dimensional cell culture.

Therefore, the objective of this study was to screen genes by DNA microarray assay that could be related to new bone formation caused by a static tensional force. The identified genes were confirmed by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

RESULTS AND DISCUSSION

The DNA microarray data showed that 2,856 genes (7.14%) were up-regulated and 2,574 genes (6.44%) were down-regulated by the static tensional force after 2 hours. When the force was applied for 12 hours, 585 genes (1.46%) were up-regulated and 857 genes (2.14%) were down-regulated. Among them, genes which are related to collagen synthesis, angio-

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genesis and bone formation were selected and listed (Table 1, 2). Considering that static tensional forces induce new bone formation *in vivo*, the role of PDL cells in new bone formation could be partially explained by this experiment. Interestingly, except for ALP, several known osteogenic genes such as bone morphogenic proteins (BMPs), osteopontin, and osteonectin

Table 1. The results of DNA microarray after the application of 2 hours static tensional force

Title	GenBank	Chromosome	Fold ratio
Alkaline phosphatase	NM_031313	2q37	2.614
Calcium/calmodulin-dependent protein kinase IV	NM_001744	5q21.3	3.212
Collagen type III alpha 1	NM_000090	2q31	3.366
Collagen type V alpha 1	NM_000093	9q34.2-q34.3	7.603
Collagen type VI alpha 1	NM_001848	21q22.3	2.654
Collagen type VI alpha 2	NM_001849	21q22.3	4.754
Elastin	BC065566	7q11.2	2.121
Fibroblast growth factor 17	NM_003867	8p21	3.141
Fibroblast growth factor 8	NM_033163	10q24	3.302
Interleukin 8	X77737	17q21q	2.039
Interleukin 11	NM_000641	19q13.3-q13.4	5.728
Matrix metallopeptidase 11 (stromelysin 3)	NM_005940	22q11.23	2.195
Matrix metallopeptidase 16 (membrane-inserted)	NM_005941	8q21	3.979
Matrix metallopeptidase 21	NM_147191	10q26.2	9.163
Phosphatidylinositol 4-kinase type 2 beta	NM_018425	10q24	4.581
Phosphoinositide-3-kinase, regulatory subunit 4	NM_014602	3q22.1	2.803
Platelet-derived growth factor alpha polypeptide	X06374	7p22	3.430
Annexin A7	NM_004034	10q21.1-q21.2	−3.115
Collagen type I alpha 2	NM_000089	7q22.1	−9.804
Collagen type IV alpha 4	NM_000092	2q35-q37	−3.096
Fibroblast growth factor 7	NM_002009	15q15-q21.1	−2.141
Fibroblast growth factor receptor 1	NM_023110	8p11.2-p11.1	−2.817
Glutaminase	NM_014905	2q32-q34	−4.255
Interleukin 7 receptor	NM_002185	5p13	−6.135
Phosphodiesterase 8B	NM_003719	5q14.1	−3.953
Retinoic acid receptor, alpha	NM_000964	17q21	−2.062
Transforming growth factor, beta 3	NM_003239	14q24	−2.915

Table 2. The results of DNA microarray after the application of 12 hours static tensional force

Title	GenBank	Chromosome	Fold ratio
Collagen type I alpha 1	NM_000088	17q21.33	2.581
Collagen type I alpha 2	NM_000089	7q22.1	2.071
Collagen type IV alpha 2	NM_001846	13q34	4.390
Collagen type V alpha 3	NM_015719	19p13.2	3.284
Glutaminase	NM_014905	2q32-q34	3.775
Interleukin 11	NM_000641	19q13.3-q13.4	3.769
Matrix metallopeptidase 2	NM_004530	16q13-q21	2.586
Platelet-derived growth factor alpha polypeptide	X06374	7p22	2.997
Elastin	BC065566	7q11.2	5.929
Phosphatidylinositol-4-phosphate 5-kinase, type I	NM_012398	19p13.3	2.611
TIMP metallopeptidase inhibitor 1	NM_003254	Xp11.3-p11.23	2.634
Vascular endothelial growth factor C	NM_005429	4q34.1-q34.3	3.202
Vitamin D (1,25- dihydroxyvitamin D3) receptor	NM_001017535	12q13.11	2.492
Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	NM_004064	12p13.1-p12	−2.667
Cyclin-dependent kinase inhibitor 2B (p15)	NM_078487	9p21	−2.188
Interleukin 6 signal transducer	CR621148	5q11	−2.959
Transforming growth factor, beta 3	NM_003239	14q24	−2.618
Interleukin 15	NM_172174	4q31	−2.045
BTG family, member 2	NM_006763	1q32	−2.066
Carboxypeptidase E	NM_001873	4q32.3	−3.215
Interleukin-1 receptor-associated kinase 4	NM_016123	12q12	−2.268
Platelet-derived growth factor receptor	NM_006206	4q11-q13	−2.392

did not show any significant changes in expression level in response to 10% static tension.

Genes related to collagen synthesis and the angiogenesis

The genes that showed higher expression after 2 hours of static tensional force are listed in Table 1 and include *calcium/calmodulin-dependent protein kinase IV, collagen type III alpha 1 (COL3A1), COL5A1, elastin, fibroblast growth factor-17 (FGF-17), FGF-8, interleukin-8 (IL-8), IL-11, matrix metalloproteinase-11 (MMP-11), MMP-16, MMP-21, phosphatidylinositol 4-kinase, phosphatidylinositol 3-kinase,* and *platelet-derived growth factor-A (PDGF-A)*. In addition, various types of collagen were increased in PDL cells by the static tensional force, including *COL1A1, COL1A2, COL4A2,* and *COL5A3* after 12 hours application of the static tensional force (Table 2). In particular, type I collagen is an important matrix protein that helps heal the alveolar bone (9) and accelerates new bone formation in the bony defect in an animal (10). However, after only 2 hours *COL1A2* and *COL4A4* were significantly decreased (Table 1). Type IV collagen is initially reduced in the tension side of PDL during orthodontic treatment but recovers and its expression gradually increased (11). The protein content of type IV collagen also increased after 72 hours of static tensional force ($P < 0.005$, Fig. 1A). For the osteoblast, although expression of COL1A1 is not affected by 4 hours of shear stress, it increase when shear stress is applied for 24 hours and static conditions for an additional 13 days (12). However, we could not measure the concentration difference of type I collagen caused by the static tensional force because the cells were grown in the type I collagen matrix. As mechanical stress might not induce collagen synthesis initially, an adaptation period might therefore be needed to allow matrix synthesis to increase, which in turn would be influenced by the character of the mechanical stress. This increased expression of genes related to collagen synthesis has beneficial effects on new bone formation.

Angiogenesis is also highly important in new bone formation (13) and its factors may be related to the activation of the osteoclast and resultant bony resorption (14). Therefore, the activation of angiogenic factors may be a double-edged sword and must be interpreted by additional factors. *PDGF* and *vascular endothelial growth factor (VEGF)* are strong angiogenic factors (15), both of which are increased in expression after the application of static tensional force (Table 1, 2). The protein level of VEGF also increased following 72 hours of static tensional force (Fig. 1A). Therefore, application of static tensional force increases genes related to angiogenesis in PDL cells. For orthodontic tooth movement, VEGF mRNA expression was found in PDL cells and VEGF concentration increased in gingival crevicular fluid (16). The expression of VEGF, highly sensitive to the mechanical stress (17), is detected in osteoblasts on the tension side (18) while induction of VEGF-A by mechanical stress can increase new bone formation (19). Therefore, the increased expression of VEGF mRNA and protein in PDL cells could explain the new bone formation on the tension side during orthodontic tooth movement. In addition, *IL-8* is a strong angiogenic factor (20) and also up-regulated by the static tensional force (Table 1).

Genes related to new bone formation

Expression of *ALP* significantly increased after 2 hours, according to the DNA microarray data (Table 1). Its expression also increased at 12 hours, but it was not significant (data not shown). However, real-time RT-PCR revealed its expression decreased after 2 hours then increased ater 12 hours (Fig. 2). ALP activity relative to the control was 1.23 ± 0.09 ($P < 0.05$) and 0.86 ± 0.14 ($P < 0.05$) at 24 hours and 72 hours, respectively (Fig. 1B). When PDL cells were treated with cyclic tension for 5 days, ALP activity decreased (7). However, a conflict is presented as other studies show that uniaxial cyclic tension increases the expression of ALP in PDL cells (8). This may be due to differing experimental conditions. During orthodontic tooth movement, ALP activity is greater in the tension site than the compression site (21). Considering that new bone formation is observed on the tension side during orthodontic tooth movement, experimental conditions that produce increased ALP activity are more likely similar to the clinical observation.

IL-6 is a strong osteoclast activator (22) that increase in PDL

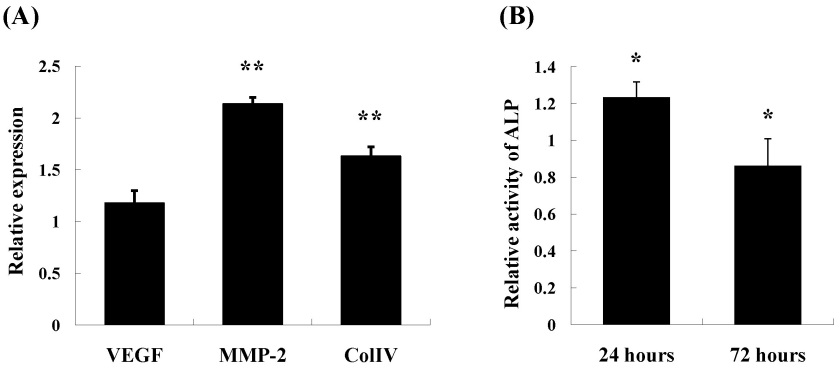

Fig. 1. Expression of type IV collagen, vascular endothelial growth factor (VEGF), matrix mettaloproteinase-2 (MMP-2), and alkaline phosphatase (ALP) activity in response to static tensional force. Primary PDL cells were three-dimensionally cultured and subjected to static tensional force for 24 or 72 hours. Medium was harvested from each samples and type IV collagen, VEGF, MMP-2 and ALP activity were measured. (A) Type IV collagen and VEGF were measured after 72 hours of tensional force. MMP-2 was measured at 24 hours. The relative activity was the fold ratio of the protein concentration to the control. (B) ALP activity was determined after 24 hours and 72 hours application of static tensional force. Each sample was triplicated and represented as mean + SD as bar. *$P < 0.05$, **$P < 0.005$.

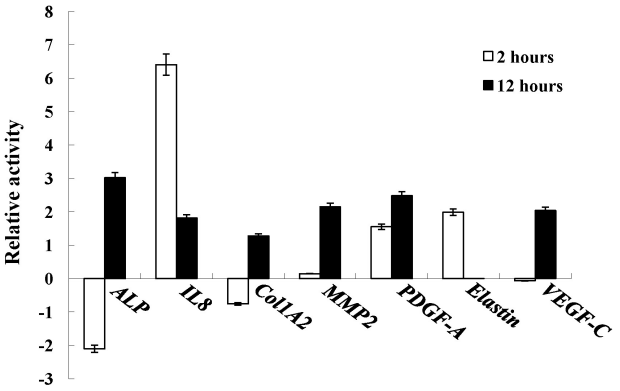

Fig. 2. Confirmation of DNA microarray results by quantitative real-time RT-PCR. Cells culture, total RNA extraction and real-time RT-PCR were performed as shown in materials and methods. White and black bars indicate the relative mRNA level of each gene after 2 hours and 12 hours of static tensional force, respectively. Control was without static tensional force. ALP: alkaline phosphatase IL8: interleukin-8, COL1A2: type I collagen, alpha 2, PDGF-A: platelet derived growth factor-A, VEGF-C: vascular endothelial growth factor-C.

cells in response to static compressive force (5, 6). *IL-6* decreased in expression after 12 hours of static tensional force (Table 2). The expression of *IL-8* was observed after 2 hours, possibly due to an early response of cells to mechanical stress. Increase of *IL-11*, an important factor in osteogenesis (23), was shown after 2 hours of static tensional force as well (Table 1).

MMPs and tissue inhibitors of metalloproteinases (TIMPs) are similarly important in new bone formation (24). The expression of MMPs and TIMPs in PDL cells was altered in response to static tensional force. Expression levels of *MMP-11, MMP-16,* and *MMP-21* increased following 2 hours of static tensional force (Table 1) while *MMP-2* and *TIMP-1* were up-regulated after 12 hours (Table 2). The protein level of MMP-2 also increased after 24 hours of static tensional force ($P < 0.005$, Fig. 1A). Interestingly, MMP-16 has been noted to be highly expressed in the reserve zone of the growth plate (25). During osteogenesis, MMP-2 is expressed in the osteoblast while TIMP-1 is related to its maturation (24). Therefore, orchestrated expression of MMPs and TIMPs might be significant, as shown in the formation of new bone (24) and tooth eruption (26).

The expression levels of other genes were also changed in response to static tensional force. *FGF-7, fibroblast growth factor receptor-1 (FGFR1), glutaminase, retinoic acid receptor alpha (RARA),* and *transforming growth factor-β3 (TGF-β3)* were all affected (Table 1, 2). However, the biological functions of these genes remain unknown during osteogenesis of PDL cells. FGF receptors can bind 17 FGFs (FGF1-17) and are important in bone growth during the developmental period (27). Controlled release of TGF-β3 can inhibit osteoblast differentiation and ALP expression (28). The expression of *TGF-β3* was inhibited after both 2 hours and 12 hours of static tensional force (Table 1, 2). *ALP* expression increased after both 2 hours (Table 1) and 12 hours (Fig. 2). Although the relationship between expression of TGF-β3 and ALP activity in PDL is unclear, it might be related to osteoblast differentiation on the tension side.

The results of real-time RT-PCR are shown in Fig. 2 after transformation by log 2 function. The relative expression of each gene is presented after normalization with *glyceraldehyde-3-phosphate dehydrogenase (GAPDH). ALP, IL-8, COL1A2, MMP-2, PDGF-A, elastin,* and *VEGF-C* were examined and the results generally corresponded to the DNA microarray assay.

In conclusion, many genes up-regulated by static tensional force are related to osteogenic processes such as matrix synthesis and angiogenesis in the three-dimensional PDL cells culture.

MATERIALS AND METHODS

Cell culture and application of static tension

Primary cultured human PDL cells were cultured as described previously (5). Cultures were washed 3 times with PBS twenty-four hours prior to application of static tension and 10 ml of serum-free medium was added to each plate. For creating the static tensional force, PDL cells were cultured in a three-dimensional collagen gel system. Collagen gel stock was synthesized from calf skin (Sigma, St Louis, MO) by melting type I collagen with 1 mM acetic acid. Static tensional force was applied via elevating the flexible bottom of the plate under the collagen gel (see supplementary Fig. 1). The tensional force resulted in a 10% length increase of the collagen gel. Cell culture under stress was performed in 37°C, 5% CO_2 for 2 hours and 12 hours. The control was also cultured under the same conditions without tensional force.

Analysis of gene expression using DNA microarray technology

After removing the culture medium, total RNA was extracted using Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH). The subsequent procedure was performed according to manufacturer's protocol. Quality of the extracted mRNA was verified by Agilent's 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). cDNA synthesis was performed based upon the extracted total RNA using a reverse transcriptase kit (Invitrogen, Inc., Carlsbad, CA). cDNA was transferred to chips and the DNA microarray was performed using a whole, human genome, oligo microarray kit that includes 40,000 genes (#G4112A, Agilent Technologies Inc.). The labeling dyes were cyanine3 and cyanine5. To stratify gene expression, intensity dependent normalization and filtration were performed using GeneSpring® software (Agilent Technologies Inc.). A scatter plot was generated that showed a subset of genes either up- or down-regulated in the static tensional force applied group. Genes displaying a more than 2-fold difference in the repeated experiment were selected as significantly changed genes.

Quantitative RT-PCR

Seven genes that were significantly up-or down-regulated were selected and real time RT-PCR was performed in order to confirm the microarray results. Real time-PCR was performed to relatively quantify the mRNA levels for genes of interest and used CYBR green PCR master mix (ABI, Foster city, CA). Sll primers except for VEGF-C were designed using Primer Express software (ABI). The primer design for VEGF-C was referenced from previously published work (29). Total RNA (3 μg) was reverse-transcribed and 200 ng of cDNA was used as template in each PCR. CYBR green PCR master mix (25 μl) and 1 μl of 10 mM specific primers were combined with template and water into a total volume of 50 μl. A negative control lacking template was also included in each assay. The design of primer is shown in supplementary data. The cycle threshold (Ct) values were determined that correspond to the PCR cycle number at which fluorescence emission in real time reaches a threshold above the base-line emission. The Ct value assigned to a particular well therefore reflects the point during the reaction at which a sufficient number of amplicons have accumulated in that well to a statistically significant point above base line. The measured value was compared to the corresponding microarray value.

ELISA and ALP activity assay

For protein analysis cells were grown in the same conditions as previously mentioned, except that cells were grown in the same medium without serum. Briefly, twenty-four hours prior to the application of static tension, cultures were washed 3 times with PBS and 10 ml of serum-free medium was added to each plate. The same amount of static tensional force was applied for 24 hours or 72 hours. Control conditions lacked any tensional force. ELISA was performed with the supernatant and the concentrations of VEGF and type IV collagen were measured at 72 hours by kits (Quantikine Immunoassay kit; R&D systems, Minneapolis, MN and Collagen IV ELISA kit; Echelon Biosciences Inc., Salt Lake City, UT). The concentration of MMP-2 was measured at 24 hours by kits (Express ELISA kit; GenScript corporation, Piscataway, NJ).

The enzyme activity of ALP was measured in accordance to previous publications (5). Briefly, cells were washed with PBS three times and then solubilized with 0.02% Triton X-100/ 0.9% NaCl and 0.02% nonident P-40. After sonification, cell lysates were centrifuged at 13,000 rpm. Collected supernatant was incubated in 96-well plate for 30 min. The reaction was stopped by adding 200 μl of 1 N NaOH. The absorbance was measured at 450 nm.

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