Expression of alkaline phosphatase in immortalized murine cementoblasts in response to compression-force

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ABSTRACT

الأهداف: اختبار قوة الضغط على إنتاج أنزيم الفوسفاتيز القلوي، بالإضافة إلى تحديد نشاط هذا الإنزيم في الخلايا الأرومية الملاطية.

الطريقة: أُجريت هذه الدراسة في معمل أمراض الفم، مستشفى طب الفم بالصين الغربية، جامعة سيشوان، شينغدو، سيشوان، الصين وذلك خلال الفترة من أكتوبر إلى ديسمبر 2010م. لقد قمنا بتعريض الخلايا الأرومية الملاطية لقوة ضغط 2000 يوسترين وذلك بتردد يصل إلى 0.5 هرتز لمدة ساعة، و3، و6، و12، و24 ساعة، وفحصنا بعد العلاج نشاط الفوسفاتيز القلوي في الخلايا. لقد قمنا باستخدام التفاعل التسلسلي المبلمر ذو الوقت الفعلي واختبار لطخة ويسترن من أجل تحليل الجينات والبروتينات بعد قوة ضغط الفوسفاتيز القلوي في الخلايا الأرومية الملاطية في أوقات مختلفة.

النتائج: لقد قامت قوة الضغط 2000 يوسترين بزيادة الفوسفاتيز القلوي في الخلايا الأرومية الملاطية وذلك في اختبار التفاعل التسلسلي المبلمر ذو الوقت الفعلي حيث وصلت الذروة إلى 12 ساعة. كما كان هناك توافقاً بين تغير إنتاج البروتينات استجابةً لقوة الضغط على إنتاج الفوسفاتيز القلوي وتغير مستويات الجينات. وقد لاحظنا تحسناً ملحوظاً في الخلايا الأرومية الملاطية خلال تطبيق هذا الضغط الميكانيكي.

خاتمة: أظهرت الدراسة دور قوة الضغط في زيادة إنتاج الفوسفاتيز القلوي في الخلايا الأرومية الملاطية. وهذا بدوره يشير إلى أن التحفيز الميكانيكي قد يؤثر على الوظيفة الخلوية لهذه الخلايا وذلك من خلال تنظيم إنتاج الفوسفاتيز القلوي الذي قد يشارك في عمليات الأيض لهذه الخلايا خلال تحرك الأسنان.

Objectives: To determine the effect of compression-force on the expression of alkaline phosphatase (ALP), and ALP activity in cementoblasts.

Methods: We performed this study in the State Key Laboratory of Oral Diseases, West China Stomatology Hospital, Sichuan University, Chengdu, Sichuan, China from October to December 2010. We exposed murine immortalized cementoblasts (OCCM-30) to 2000-μstrain compression-force at a frequency of 0.5 Hz for 1, 3, 6, 12, and 24 hours. We assayed the cellular ALP activity after the treatments. We used real-time polymerase chain reaction (RT-PCR) and western blot to examine the gene and protein expression of ALP in the OCCM-30 cells at each time point.

Results: Two-thousand μstrain compressive force significantly up-regulated the mRNA expression of ALP in OCCM-30 cells, which reached a peak at 12 hours loading, and the protein expression change of ALP in response to compression-force was consistent with the variation of gene level. We also noted marked enhancement of ALP activity in OCCM-30 cells during the application of mechanical stress.

Conclusion: The compression-force increased the expression of ALP in OCCM-30 cells, suggesting that mechanical stimulation may affect the cellular function of cementoblasts by regulating ALP expression, which may participate in cementum metabolism during orthodontic tooth movement.

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Cementum is a unique mineralized connective tissue covering the roots of teeth. Despite a number of similarities that cementum shares with bone,[1] cementum is often more resistant than bone in response to mechanical environmental changes,[2,3] which is also known as a physiological basis of orthodontic tooth movement. However, orthodontic force application can sometimes evoke excessive cementum resorption, even eventually developing to dentinal or apical root resorption.[4,5] Then, cementoblasts, root surface lining cells, can be activated to form reparative cementum. Although it has been realized that cementoblasts participate in the healing process of orthodontically induced root resorption,[6-8] the exact mechanisms or factors involved in regulating cementum-formation of cementoblasts remain undefined. Previous studies have proved that mechanical stress regulated bone sialoprotein (BSP) and osteopontin (OPN) expression in cementoblasts.[9,10] It is thus likely that mechanical stress evokes biochemical responses in cementoblasts, which may consequently affect cementum metabolism.

It is generally accepted that alkaline phosphatase (ALP) is involved in the process of calcification in various mineralizing tissues.[11] Beertsen and Everts[12] put forward the view that ALP participates in cementum formation, which has been supported by later research demonstrating the positive correlation between ALP activity and the thickness of cementum.[13] More importantly, the dramatic inhibitory effect of tissue-non-specific alkaline phosphatase (TNAP) deficiency on cementum formation observed in patients with hypophosphatasia and TNAP-null mice ($Akp2^{-/-}$) has drawn the attention of many researchers,[14-16] providing deep insights into the critical role of ALP in cementogenesis. Although some studies have reported little or no ALP expression in cementoblasts,[17,18] many other scholars think that cementoblasts could express ALP.[19-21] Several studies have revealed the effects of mechanical forces on ALP activity in MC3T3-E1 cells in vitro and mouse periodontal osteoblasts in vivo.[22,23] However, the ALP expression in cementoblasts in response to mechanical stimulation has not been clarified.

Since ALP is considered important for cementum formation, it is plausible to postulate that ALP may be involved in the repair capability of cementoblasts during the mechanical stress-induced root resorption. The objective of this study was to examine the effect of mechanical compression-stress on ALP activity and its expression at both protein and mRNA levels in cementoblasts (OCCM-30 cells). As such, the results of the current study will be helpful in understanding the reparative process following orthodontic root resorption, which is essential for further improvement of orthodontic procedures.

Methods.

Cell culture. The immortalized murine cementoblasts (OCCM-30), characterized as highly differentiated cementoblasts,[24] were kindly provided by Professor Somerman (Washington, USA) as a gift. The OCCM-30 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Hali Biotechnology Company, Chengdu, China), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine at 37°C in a humidified atmosphere of 5% CO_2 in air.

Application of mechanical force. External mechanical stimulation was achieved by a self-made 4-point bending system, as described in previous studies.[9,25] The OCCM-30 cells were seeded into the force-loading plates at a density of $4x10^5$ cells/ml, with a total 2 ml in each plate. Cyclic uniaxial compressive force was applied on the plates at a frequency of 0.5 Hz. Cells were subjected to the stress at 2,000 μstrain for one, 3, 6, 12, and 24 hours. All the loading processes were completed in a humidified atmosphere of 95% air and 5% CO_2. Cells in the control group were cultured on similar plates and maintained in the same incubator without mechanical stress loading. Experiments were repeated 3 times with comparable results.

Assay of alkaline phosphatase (ALP) activity. At defined time points, the ALP activity of treated and untreated OCCM-30 cells was detected using an ALP assay kit (BoDing, Beijing, China). In brief, cells were washed 3 times with PBS, and lysed in non-denaturant conditions by using 0.1% Triton X-100. After centrifugation, a reaction buffer, pH 7.4, composed of 100 mM glycine buffer, and 2 mM $MgCl_2$ in distilled water was added with 16 mM p-nitrophenyl phosphate to the cell supernatants, and the reaction was stopped with 0.1 M NaOH. Then ALP was measured spectrophotometrically at a 405-nm wavelength.

Real-time RT-PCR analysis. Cells were harvested immediately after loading finished for RNA extraction. By using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), the OCCM-30 cells' RNA was split, collected, purified, and extracted according to the manufacturer's protocol. The cDNA synthesis and PCR procedures were performed using the Takara RT-PCR kit (Takara, Otsu, Shiga, Japan). Specifically, the overall ALP forward primer was 5'-CCCCCCGTGGCAACTCTATCTT-3' and the reverse primer was 5'-GTAGTTCTGCTCGTGGACGCCG-3', yielding an amplicon of 272 bp. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward primer was 5'-CCTCAAGATTGTCAGCAAT-3' and the reverse primer was 5'-CCATCCACAGTCTTCTGGGT-3', yielding an amplicon of 141 bp. The GAPDH gene expression was chosen for normalization of

each sample (housekeeper gene). The amplification profile (temperature[°C]/time[s]) was 94/120 (initial denaturation), 94/20 (denaturation), optimal annealing condition 58/20, and 60/30 (extension), all for 45 cycles. The PCR processes were repeated 3 times.

Western blotting analysis. The ALP protein expression was evaluated by Western blot analysis. The OCCM-30 cells were collected and rinsed 3 times with ice-cold PBS, and then resuspended in lysis buffer (50mM Tris–HCl, 150mM NaCl, 1% Triton X-100, 1mM EDTA, 0.5 mM phenyl-methanesulfonyl fluoride, 10% glycerol) supplemented with proteinase inhibitors. The protein concentration of cell lysates was analyzed by BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins (20μg) were loaded onto 10% SDS-polyacrylamide gels (Amersham, New Jersey, USA) and electrotransferred onto Polyvinylidene fluoride (PVDF) membranes (Roche, Mannheim, Germany). After being blocked in 5% nonfat dry milk in TBS-T (TRIS-buffered saline and 0.1% Tween-20) for one hour at room temperature, membranes were incubated at 4°C overnight with rabbit anti-ALP (1:500 dilution; Abcam, Cambridge, UK) and rabbit anti-β-actin (1:500 dilution; BIOS, Arizona, USA). Specific proteins were revealed by horseradish peroxidase (HRP)-conjugated secondary antibodies (dilution 1:1000; Milipore, California, USA). The immunoreactive bands were detected by enhanced chemiluminescence (Santa Cruz, California, USA). The density of bands was analyzed using Quantity One 1-D analysis software on a Chemi Doc XRS System (Bio-Rad, California,USA), and normalized to that of control β-actin.

Statistical analysis. Data were expressed as mean ± standard error of the mean and analyzed by one-way ANOVA based on 3 independent experiments. If statistical differences were detected, multiple comparisons using the Student-Newman-Keuls (SNK) test were carried out. We used the Statistical Package for Social Sciences 11.5 (SPSS, Chicago, IL, USA) software for analyses, and the confidence level was 95%, and $p<0.05$ was considered to be statistically significant.

Results.

Alkaline phosphatase activity. As shown in **Figure 1**, we found that ALP activity increased significantly ($p=0.007$) at one hour of 2,000-μstrain compression-force stimulation, then kept relatively stable from one hour to 6 hours, and reached the peak level ($p<0.001$) at 12 hours. Despite a decrease after 24 hours, the overall level of ALP activity showed a rise tendency.

Effect of mechanical force on mRNA expression of alkaline phosphatase. During the application of mechanical stress, the time-course profile of mRNA expression of ALP was examined by real-time PCR analysis. As shown in **Figure 2**, after the loading of 2,000-μstrain compression, the expression of ALP mRNA was found to present an increasing tendency. At 3 hours of mechanical stimulation, the expression of ALP mRNA increased markedly ($p=0.026$), followed by a slight drop, then gradually up-regulated to the peak at 12 hours ($p=0.018$). After 24 hours, there was a decrease in mRNA expression of ALP, but the statistical significance ($p=0.039$) was still noted compared with control samples.

Effect of mechanical force on expression of alkaline phosphatase protein. Based on western blot analysis, the immunoreactive bands of ALP and β-actin protein were observed clearly with strong specificity (**Figure 3a**). Through comparative analysis of gray intensity of protein bands, ALP protein was found to be expressed weakly in normally cultured OCCM-30 cells, but increased markedly after one hour of 2,000-μstrain compression-force loading, and then maintained high-level expression

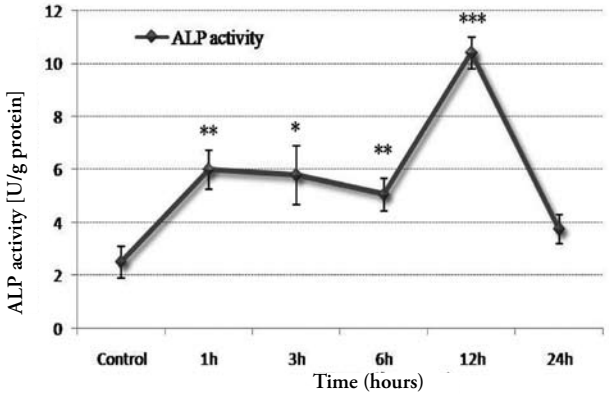

Figure 1 - Effects of mechanical compressive stress on alkaline phosphatase (ALP) activity (compared with controls $p=0.007$ for one hour, $p=0.008$ for 6 hours, $^{***}p<0.001$)

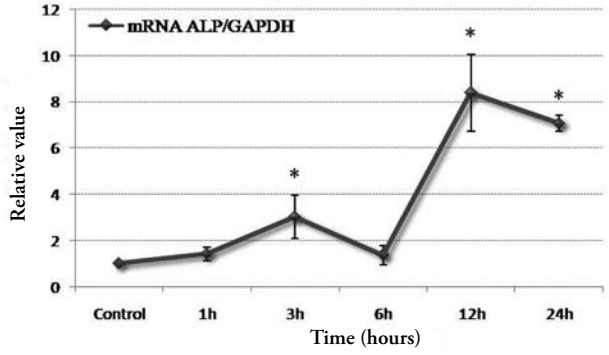

Figure 2 - Effects of mechanical compressive stress on alkaline phosphatase mRNA expression (compared with controls: $p=0.026$ for 3 hours, $p=0.018$ for 12 hours, $p=0.039$ for 24 hours). mRNA - messenger ribonucleic acid, ALP - alkaline phosphatase, GAPDH - glyceraldehyde 3-phosphate dehydrogenase.

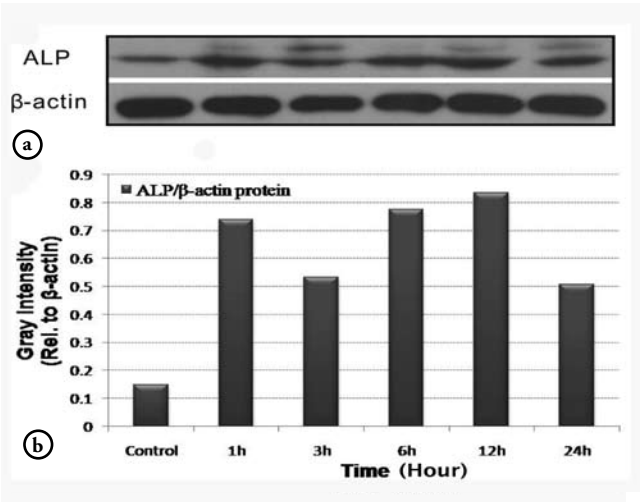

Figure 3 - Effects of mechanical compressive stress on alkaline phosphatase (ALP) protein expression: **a**) the immunoreactive bands of ALP and β-actin protein, **b**) time-course changes of gray intensity of ALP protein.

although it fell back a little at 3 hours and 24 hours (**Figure 3b**).

Discussion. Our findings suggest that 2000-μstrain compression-force increased the ALP activity of OCCM-30 cells. Further, the finding that the mRNA and protein levels of ALP in the cells were up-regulated in response to compression-force is mostly consistent with our finding of enhancement of ALP activity in response to this force.

To simulate mechanical environment in vitro, a uniaxial compressive force at a magnitude of 2000 μstrain was produced by a cyclic four-point bending system, which has been described previously[26] to be feasible for generating mechanical stress at a physiological level with good repeatability and strong controllability. Strains less than 2000 μstrain have been reported to occur in bone cells as a result of applied mechanical stresses under physiological conditions.[27,28] Fermor et al[29] demonstrated in vitro that osteoblast-like cells exposed to the stretch of 1600 μstrain had higher proliferative activity than those under forces at other magnitudes, and strains above 4000 μstrain are more relevant to injury and fracture healing. Meyer et al[30,31] showed that strains of 2000 μstrain promoted the proliferation and osteogenic differentiation of osteoblast-like cells and positively regulated the assembly and mineralization of collagen during distraction osteogenesis, while the hyperphysiological strains led to undesirable results. Also, our previous studies have found that cementoblast-like cells could grow well after the loading of forces ranging from 1000 μstrain to 4000 μstrain, and especially when the force of 2000 μstrain was applied, the cells possessed high proliferation activity and kept normal morphology.[9] Thus, this information suggests that 2000-μstrain would be an appropriate parameter for investigating physiological mechanostimulation of cells in vitro.

Physiological optimum light force is always advocated in orthodontic treatment to make tooth movement more efficient.[32,33] However, even with light forces, root resorption is unavoidable within the compressed areas.[5,32] When orthodontic forces are applied, there is usually an attack on the cementum of the root, but repair of the cementum in which cementoblasts participate also occurs. Therefore, in this study, we selected the force of 2000 μstrain, which is the optimum force in the in vitro model to imitate the clinical optimum orthodontic force. Moreover, the force was compressive to simulate the mechanical microenvironment on the pressure side where resorption occurs in vivo. In addition, in order to reduce the effect of fluid shear stress produced by medium fluctuations on the cells, the cyclic uniaxial force is applied at low frequency of 0.5 Hz.

As one of the most frequently used biochemical markers to identify cells with osteogenic potential, ALP is expressed on the cell membrane of osteoblasts and odontoblasts, and is also concentrated on the membranes of the matrix vesicles released by these cells.[34] However, it is still controversial whether cementoblasts should express ALP or not. Some researchers reported that ALP activity in cementoblasts was weak or lacking.[17,18] But more studies showed that cementoblasts could express ALP,[19-21] and it has been found that human cementoblasts have a high ALP activity showing an active capacity for mineralization.[35] Consistent with this idea, our results demonstrated the positive expression of ALP in OCCM-30 cells.

Alkaline phosphatase is a glycoprotein involved in processes leading to mineral formation in tissues like bone and cementum. Thought to be a biomineralization promoter, ALP hydrolyzes pyrophosphate, which is an inhibitor of hydroxyapatite formation, and provides inorganic phosphate for the formation of hydroxyapatite.[36,37] There is some evidence that ALP participates in cementum formation. For example, hypophosphatasia, a disease caused by loss-of-function mutation of the gene that encodes the tissue nonspecific isoenzyme of alkaline phosphatase (TNAP), results in severely reduced or the lack of cementum formation, and subsequently the loss of teeth at an early age,[38] which has been successfully mimicked in TNAP-null mice ($Akp2^{-/-}$) manifesting defective cementum.[16,39] Moreover, previous study[43] recently reported that the enzyme-replacement therapy by daily injections of human TNAP prevented hypomineralization of cementum in $Akp2^{-/-}$ mice, suggesting that ALP plays a

critical regulatory role in the formation of cementum. In our study, ALP activity and the expression levels of ALP mRNA and protein in OCCM-30 cells increased significantly during 24 hours of mechanical stimulation in vitro, indicating that ALP might be an important target for mechanical signals in cementoblasts. As it is widely held that ALP is important in the initiation of mineralization processes,[40] we could deduce that mechanical stimuli may promote the biomineralization of cementoblasts by up-regulating ALP expression. However, as the effects of compressive force on in vitro biomineralization of OCCM 30 cells have not been investigated in this study, interpretation of how the cells are affected is limited. Therefore, further studies are required to test this hypothesis. The mechanism of increased ALP expression in OCCM-30 cells in response to compression-force was not determined in this experiment. Previous studies have suggested that prostaglandin E_2 (PGE_2) stimulated ALP activity in cementoblasts,[41] while PGE_2 and EP4 (PGE receptor) agonists led to downregulation of mineralized nodule formation and ALP activity in OCCM-30 cells.[42] Besides, it has been reported that compressive force stimulated PGE_2 production and EP2 and/or EP4 expression in osteoblasts.[43] Because cementoblasts share many molecular properties with osteoblasts,[44] there is a possibility that cementoblasts would also release PGE_2 in response to the compression-force, thus up-regulating its ALP expression, which needs further investigation. Mechanical loads should be differentiated into compression and tension type. According to our previous study, there was no significant difference between tensile and compressive stresses in the influence on cell proliferation and BSP mRNA expression in OCCM-30 cells.[9] Chan and Darendeliler[45] also showed that cellular changes in vivo were similar between the compressive and tensile sides in tooth movement. So it is possible that cementoblasts respond similarly to mechanical tensile and compressive stresses. However, further tensile loading experiments are required to confirm a similar effect on ALP expression.

In summary, the ALP expression in OCCM-30 cells is increased in response to compression-force, suggesting that mechanical stresses such as orthodontic forces could modulate ALP activity and expression in cementoblasts, which in turn may affect cementum metabolism in orthodontic treatment, depending on the functional role of ALP in cementogenesis. The findings provide a novel perspective into the molecular mechanisms in the reactions of cementum during orthodontic therapy.

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