Original Article
Expression and function of osteogenic genes runt-related transcription factor 2 and osterix in orthodontic tooth movement in rats

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Received July 31, 2015; Accepted August 28, 2015; Epub September 1, 2015; Published September 15, 2015

Abstract: Objective: To investigate the expression and function of osteogenic genes osterix (OSX) and runt-related transcription factor 2 (RUNX2) in the rat periodontal tissues under orthodontic force for the remodeling of the periodontal tissues. Methods: 24 Wistar rats were randomly divided into 4 groups of orthodontic tooth movements for 1, 3, and 7 days (experimental groups) and control group (without orthodontic force). The expression of RUNX2 and OSX in the periodontal tissues was analyzed using real time PCR for mRNA and Western blot analysis for protein. The data were also analyzed for involvement of the two genes in signal pathways using bioinformatics tools. Results: The mRNA levels of RUNX2 and OSX increased in the periodontal tissues after subjected to the orthodontic force for 1 to 7 days, with the highest level occurring at day 7. The relative expression levels of RUNX2 and OSX mRNA were 1.85±0.12, 304±0.06 and 4.16±0.068, and 1.52±0.09, 1.83±0.03 and 2.56±0.06 at day 1, 3 and 7, respectively. The results of Western blot analysis were consistent with the mRNA results. Conclusion: In orthodontic tooth movement, the expression of RUNX2 and OSX was upregulated as a result of external stimulation, suggesting that the two genes is involved in periodontal tissue remodeling and plays an important role in periodontal tissue remodeling.

Keywords: Orthodontic force, periodontal tissue, osteogenic gene

Introduction

Orthodontic treatment applies orthodontic force to the tooth through a variety of appliances to treat wrong jaw deformity and can improve occlusion to address the esthetic and functional problems. However, its longer treatment period has limited its clinical application. Therefore, effective control of tooth movement and shortening of the treatment cycle are important clinical issues to be addressed for better treatment [1, 2].

Orthodontic tooth movement is a complex biological and mechanical process involving a variety of molecules. Orthodontic force exerted on the periodontal tissue can activate osteoblast and osteoclast, resulting in their resorption and deposition in the alveolar bone, and stimulate the growth of cells and regeneration of collagen [3]. More specifically, the cells can be induced by the mechanical force to degenerate collagen on the pressed side and enhance bone absorption. Under the force, cells in the extended periodontal begin to proliferate and bone tissue begins to deposit, resulting in bone remodeling and tooth movement. Orthodontic tooth movement depends on the rebuilding of alveolar bone. The formation of osteoclasts is the prerequisite of alveolar bone remodeling. Under pressure stimulation, osteoclast generates osteoclastic reaction. It has been shown that the tooth movement can be effectively regulated by controlling the differentiation and function of osteoclast [4]. Therefore, a better understanding of molecular mechanism underlying the growth and differentiation of osteoclast is important for elucidating the molecular mechanism for orthodontic tooth movement.

Many studies have investigated how mechanical stimuli are transformed into biochemical signals to activate signaling pathways to regulate the synthesis of relevant proteins [5, 6]. Early work shows that the function of cementoblast may be altered when stimulated by me-
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Table 1. Primer used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession NO.</th>
<th>Primer sequence (5'-3')</th>
<th>Length of expected product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSX</td>
<td>NM_130458.3</td>
<td>For: GGAAGGAGGGCAAAAGAAGC</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: CCCCTAGGCACATGGAGC</td>
<td></td>
</tr>
<tr>
<td>RUNX2</td>
<td>XM_008262992.1</td>
<td>For: GACTGTGGTACCGTCATGCC</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: ACTTGGTTTTTCATAACAGCGGA</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM0002046</td>
<td>For: CCTCAAGATGTCAAGCAAT</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: ACCACAGTCCATGCGCATC</td>
<td></td>
</tr>
</tbody>
</table>

OSX: Transcription factor Sp7; RUNX2: Runt-related transcription factor-2; GAPDH, glyceraldehyde phosphate dehydrogenase; For, forward; Rev, reverse.

Several growth factors have been shown to be involved in the orthodontic tooth movement, such as insulin-like growth factor, transforming growth factor, fibroblast growth factor, bone morphogenetic protein [8, 9]. Runt-related transcription factor-2 (RUNX2) is a multifunctional transcriptional factor and expressed in the whole process of osteoblast cell differentiation [10]. Osterix (OSX) is zinc finger transcriptional factor and also indispensable regulator for the growth and differentiation of osteoblast and development of bone tissue [11]. However, little is known about their expression in periodontal tissues during orthodontic tooth movement.

In this study, we used animal models to investigate the expression of the two genes in periodontal tissues and analyzed their involvement in signaling pathways to elucidate their possible functions in orthodontic tooth movement.

Materials and methods

Animal model and orthodontic treatment

Male Wistar rats (SPF, weighing 250±50 g), purchased from Silaike Experimental Animal Co., Ltd. (Shanghai, China), were used for the study. The rats were fed with standard pellet feed in animal cages (5 per cage) with free access to drinking water and feed during the experiments. After 7 days of adaptive feeding, the rats were randomly divided to establish orthodontic tooth movement model as described [12]. Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 mL/kg), and shallow concaves of about 0.5-1.0 mm deep were cut on the axial plane angle in the first molar of left upper jaw and on the side of labial surface of the incisors in left and right upper jaws using fine emery bur using a low speed dental turbine. The shallow concaves were used to fasten the orthodontic stainless steel ligature wire of 0.25 mm in diameter. One end of the wire was tied to the neck of the first molar and the other end was attached to nickel titanium orthodontic spring (IMD, Inc, Shanghai, China) to provide orthodontic force of 40 g. In experimental groups, the orthodontic force was maintained for 1, 3 and 7 days, while in control, the same orthodontic appliance was used without orthodontic force. Six rats were used in each treatment. The experimental protocols were approved by the Ethics Committee on Laboratory Animal Use, Liaocheng People’s Hospital.

Periodontal tissue

Rats were sacrificed by cervical dislocation, and tissues surrounding the first molar were taken, washed in pre-chilled PBS buffer and snap-frozen in liquid nitrogen.

Real-time PCR

Total RNA was extracted from the frozen samples using a RNA extract kit (Qiagen, USA) according to the manufacturer’s instructions and reversely transcribed into cDNA using a reverse transcription kit (Applied Biosystems, USA) according to the manufacturer’s instructions and quantified using real-time PCR using gene-specific primers (Table 1) and the 2(-Delta Delta C(T)) method [13]. Glyceraldehyde phosphate dehydrogenase (GAPDH) gene was used as internal reference for the quantification. All determinations were repeated three times. The RT-PCR reaction contained 5 µL SsoAdvanced SYBR Green Super mix (Bio-Rad, USA), 0.3-0.45 µL each of primers (300-450 nM), 100 ng cDNA template and nuclease-free water to fill up to a total reaction volume of 10 µL, and performed according to the manufacturer’s protocols (Invitrogen, USA).
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Western blot analysis

Total protein was extracted using a lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA) supplemented with a protease inhibitor cocktail kit and a phosphatase inhibitor cocktail kit (Hoffman-La Roche Ltd., Basel, Switzerland). The protein extracts were loaded, size-fractionated by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA). After blocking, the membranes were incubated with the primary monoclonal antibodies against RUNX2 and OSX (Santa Cruz Biotechnology Inc., Dallas, TX, USA) and rabbit anti-β-actin (Proteintech Group Inc., Chicago, IL, USA) at 4°C overnight. Protein expression was determined using horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies, followed by detection using enhanced chemiluminescence (EMD Millipore). Band intensity was visualized using a JS-1035 image analysis scanning system (Shanghai Peiqing Science & Technology, Co., Ltd., Shanghai, China), and quantitatively analyzed using Quantity one v4.62.

Bioinformatics analysis

Online analysis tools STRING9.05 (http://string-db.org) and KEGG pathway database (http://www.genome.jp) were used to identify the signaling pathways that Runx2 and OSX participate and proteins they interact with.

Statistical analysis

Experimental data were analyzed using SPSS16.0 statistical software for statistical analysis. The measurement data were expressed as mean ± SD. Means between the two groups were compared using Student-t test and value was considered as significant or highly significant if \( P < 0.05 \), or < 0.01.

Results

OSX and RUNX2 expression at mRNA level

Expression of the OSX and RUNX2 genes were measured using RT-PCR in the periodontal tissues after different days of orthodontic movement treatment. The results showed that compared with rats applied no orthodontic force (control), the expression of the two genes were up-regulated at all sampling times (day 1, 3 and 7) and increased with increasing treatment time with the highest levels occurring on day 7 (Figure 1). One other hand, the expression of the two genes did not change in the control group over the experimental period (\( P > 0.05 \)) (Figure 1). As a result, the difference in the expression levels between the experimental and control groups increased over the treatment period (Figure 1). The relative quantities of RUNX2 and OSX mRNA in the experiment groups were 1.85±0.12, 3.04±0.06 and 4.16±0.068, and 1.52±0.09, 1.83±0.03 and 2.56±0.06, at day 1, 3 and 7 following the orthodontic treatment, respectively.

Figure 1. Relative mRNA levels of the RUNX2 (A) and OSX (B) genes determined by RT-PCR. Control, rats not applied with orthodontic force; treatment, rats applied with orthodontic force.
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OSX and RUNX2 expression at protein level

We then analyzed the expression of the two genes at protein level in the periodontal tissue using Western blot analysis. The blot and quantitative analysis results showed that the expression of the two genes at protein level increased with increasing treatment times and reached the highest levels at day 7 (Figure 2A and 2B).

Bioinformatics analysis

Search with STRING9.05 was made for Runx2 and OSX. The results showed that a number of proteins are interacting with Runx2 and OSX as network signal molecules, including WW domain containing transcription regulator 1 (Wwtr1); SRY box containing gene 9 (Sox9); Smad family member 3 (Smad3); runt related transcription factor 3 (RUNX3); homeobox msh-like 2 (Msx2) (Figure 3). Among them Sox9, Msx2 and Run3 are related to the formation of osteoblasts. KEGG Pathway database showed that RUNX2 and OSX are involved in a number of pathways that regulate the differentiation of osteoblast.

Discussion

Mechanical force in orthodontic tooth movement has been shown to bring a number of bone cell biological activities through signal transduction, resulting in alveolar bone resorption and deposition, and causing the remodeling of periodontal tissues [1, 2, 14]. This process requires the participation of osteoclasts and cementoblast, the latter can not only participate in the repair of root through the synthesis of new cementum, but also regulate osteoclast differentiation and absorption by expressing regulatory proteins [3, 15-19].
Runx2 is osteoblast specific transcriptional factor that activates and initiates the differentiation of bone marrow mesenchymal cells into osteoblasts and regulate the maturation of osteoblasts; it also regulates the expression osteoprotegerin [20, 21]. OSX is a zinc finger transcriptional factor and an important regulatory factor in the growth and differentiation of osteoblasts and the development of bone tissue. In this study, we used orthodontic tooth movement (OTM) animal model [22] to study the expression of the two genes for their role in periodontal tissue rebuilding process.

The experimental results showed that the orthodontic force upregulated the expression of Runx2 and OSX at mRNA and protein level over the 7 day experiment period compared with the control group; and the upregulation increased with increase in experimental time and reached the highest at the end of study. On other hand, there was no change in RUNX2 and OSX expression in rats not subjected to the orthodontic force treatment. These findings suggest that RUNX2 and OSX may be involved in the early response of bone cells to mechanical signal.

Fibroblasts are the pluripotent cells in periodontal tissue, and can differentiate into osteoblast like cells by expressing proteins that confer osteoblast phenotype and functions under external mechanical forces. In this process, osteoblast specific transcriptional factor plays a central role [23]. Biologically, the upregulation of RUNX2 and OSX expression by the mechanical stimulation may activate and initiate the differentiation of the bone marrow mesenchymal stem cells into bone cells and regulate the maturation of bone cells. Furthermore, RUNX2 may regulate the expression of osteocalcin to promote the maturation of osteoblast. Thus, RUNX2 is likely playing key roles in the regulation of bone cell differentiation.

Analysis of interaction of RUNX2 with other proteins in signaling pathways showed that they interact directly or indirectly with a number of molecules involved in bone remodeling, such as Run3, Sox9 and Msx2, forming a signaling network (Figure 3). In the osteoblast differentiation and bone formation, RUNX2 as a marker for osteoblast and transcriptional factor with the runt domain can bind to the core regions in a number of enhancers and promoters. For example, RUNX2 is shown to interact with Sox9 to play an important role in cartilage differentiation [24]. Msx2 also plays regulatory role in the osteoblast differentiation. For example, Shirakabe et al showed that it acts directly with RUNX2 in an immunoprecipitation assay [25]. It would be our next topic to elucidate how RUNX2 and OSX participate in the regulation of the complex networks.

Acknowledgements

This study was supported by Shandong Province Natural Science Fund (ZR2014HL052) and Shandong Province Medical Science and Technology Development Plan Projects (2014 ws-0048).

Disclosure of conflict of interest

None.

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References

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