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Factors regulating condylar cartilage growth under repeated load application

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1. ABSTRACT

Mechanical loading can influence the biological behavior of the bone-associated cells leading to adaptive changes in skeletal mass and architecture. SOX9 and PTHrP genes are known to regulate chondrocyte differentiation and delay maturation, ultimately control the endochondral bone formation. To investigate the effects of repeated mechanical loading on bone, 280 Sprague-Dawley rats were used in this experiment. The animals were randomly allocated into experimental and control groups. Repeated mechanical loading was applied through a bite-jumping device in the experimental group. The experimental animals were sacrificed on 10 different time points together with the matched control. Total RNA was extracted from the mandibular condylar cartilage for PTHrP and SOX9 genes quantification using real-time RT-PCR. Results showed that PTHrP expression was increased and reached a peak level on the seventh day after mechanical loading was given. Repeated mechanical loading triggered a significant increase of PTHrP expression leading to another peak increment. The expression of SOX9 was highly correlated with the PTHrP expression, and its pattern of expression was similar to that of PTHrP after repeated mechanical loading. In conclusions, repeated mechanical loading on the condyle triggers the expression of PTHrP and SOX9, which in turn promotes condylar cartilage growth.

2. INTRODUCTION

Bone is a highly specialized connective tissue, the extracellular matrix is mineralized given its rigidity, providing support in vertebrates and constantly exposed to physical forces. These forces can influence the biological behavior of the bone-associated cells, altering their phenotype, gene expression, paracrine or autocrine factor secretion, etc. ultimately leading to adaptive changes in skeletal mass and architecture (1-3). A number of experimental models have been developed over the years to study the effects of local mechanical loading on bone formation. One of the commonly used models is the axial compressive loading of the rat ulna (4-7). In this model, the forearm is held in cups between the flexed carpus and the olecranon. The ulna is then loaded through the carpal joint, and overlying soft tissues.

Another interesting loading model, which is widely used as well, is bending of the long bone *in vivo* (3). Two-point contacts are made with a certain distance on one surface of the long bone, and then load is applied on the other side in between resulting in bone bending. Bending load can be applied in the medial-lateral direction by either four-point bending jig (3, 8-10) or three-points bending jig (11) causing compression on one surface of the long bone and tension on the other surface.

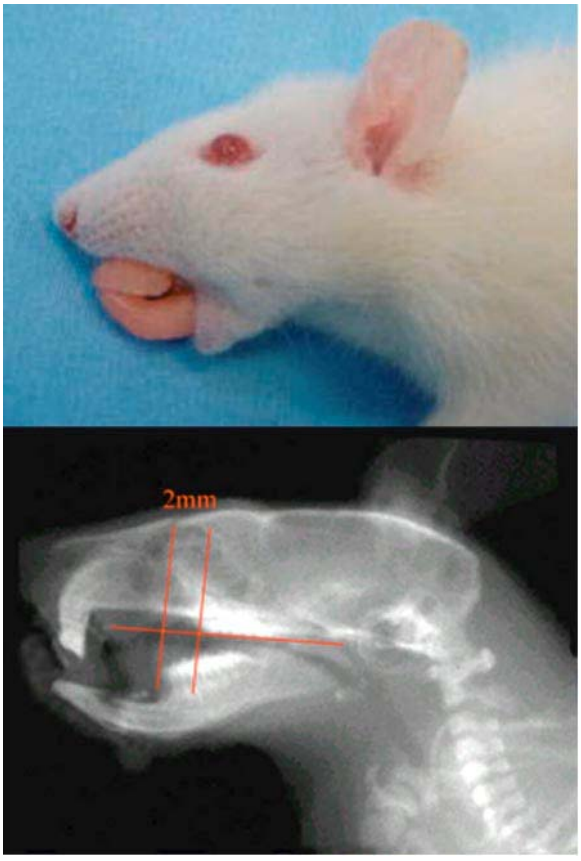

Figure 1. Intra-oral appliance used for mechanical tensile strength application at the mandibular condyle.

Physical stress application as tension was less frequently used in the study of mechanical stimulated bone adaptation. It is applied mainly on the growth plate (12, 13) or on distraction osteogenesis studies (14-17). Rabie and co-workers (18) earlier developed an experimental model which allows tensile mechanical strain to be applied on the mandible of rats. The set-up involves an intra-oral device that positions the mandible forward, therefore, generating tension at the condyle and the glenoid fossa.

Osteogenic response to mechanical loading involves several stages; in endochondral bone formation cartilage was formed as the template ahead of bone formation. Evaluation of the chondrogenic gene expression pattern allows us to understand how these genes are coordinated under physical force, subsequently leading to adaptive bone changes. Earlier, we reported that repeated mechanical loading led to enhanced condylar growth (19, 20). We also reported that mechanical loading led to a significant increase in the expression of PTHrP (21), which delayed cartilage cells maturation and endowed the condyle with more potential to buildup cartilage. This increase in PTHrP was associated with the increase of new chondrocyte populations. Furthermore, it was documented that PTHrP up-regulated SOX9 transcription (22), which has been shown to promote differentiation of mesenchymal cells into chondroblasts in the mandibular condyle (23). It is convincible that repeated mechanical load applications through mandibular advancement in a stepwise manner could re-trigger the expression of these factors and lead to further growth. Therefore, the goal of this study is to investigate the effects of repeated tensile strain on chondrogenic gene expression pattern under mechanical strain induced by stepwise mandibular forward positioning, specifically the expression of PTHrP mRNA and SOX9 mRNA using real-time RT-PCR.

3. MATERIALS AND METHODS

3.1 Experimental Design

The present study was approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR 803-03) of The University of Hong Kong. Two hundred and eighty, 5-week-old female Sprague-Dawley rats were used in this study, they were randomly allocated into control and experimental groups. The rats of the experimental group were fitted with the bite-jumping appliance described by Xiong *et al.* (2004) at day 0 of the experiment (Figure 1). This appliance maintained a continuous mandibular forward positioning of 2mm in the experimental group. After that, the rats in the experimental group received another mandibular advancement by 2mm at day 30. For the rats in the control group, no appliance was fitted. Fourteen rats from each group of animals were sacrificed at day 3, 7 14, 21, 30, 33, 37, 44, 51 and 60 of the experiment. The condylar cartilage was carefully separated from the underlying bone under a dissecting microscope with the protection from the RNAlater™ (Qiagen).

3.2 Total RNA Extraction and cDNA Synthesis

Total RNA extraction of the samples was performed using RNeasy® Fibrous Tissue Midi Kit (Qiagen) according to the manufacturer's instructions. This spin column system contains a proteinase K digestion of the fibrous protein and a DNase treatment of DNA contaminants removal. The integrity of the RNA was determined by 2100 Bioanalyzer (Agilent Technologies) and the concentration of the total RNA was quantified spectrophotometrically at 260 nm. The isolated total RNA of each sample was reverse transcribed using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) followed the manufacturer's instructions. The reaction used 2 µg of total RNA, 2 µl of random hexamer, 1 µl of 10 mM dNTP mix, 2 µl of 10X RT buffer, 4 µl of 2 5mM MgCl2, 2 µl of 0.1 M DTT, 1 µl RNaseOUT™, and 1 µl of SuperScript™ II RT. The reverse transcription process was carried out in PCR Thermal Cycler Dice (Takara).

3.3 Real-time Polymerase Chain Reaction

The PCR amplifications were performed using iCycler iQ™ Real-Time PCR Detection System (Bio-Rad). A total volume of 25 µl reaction mixture containing 1 µl of cDNA sample, 2.5 µl of 10X PCR buffer (Invitrogen), 1.6 µl of 50 mM MgCl2, 1 µl of dNTP mix, 0.4 µl of 10 µM sense and antisense primer, 0.1 µl of Taq DNA Polymerase Recombinant (Invitrogen) and 1.25 µl of 10X SYBR® Green I (Molecular Probes) was used. Amplification of cDNA included an initial denaturation at

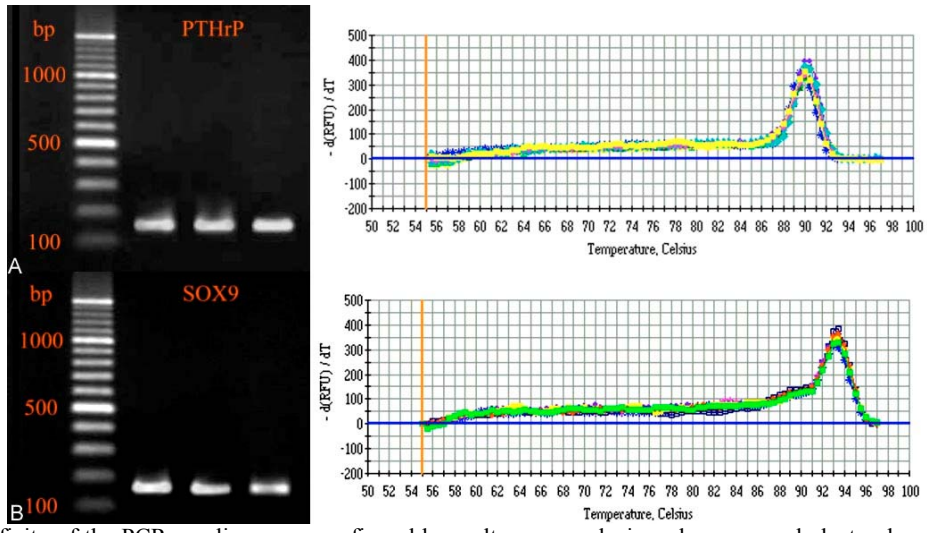

Figure 2. Specificity of the PCR amplicon was confirmed by melt curve analysis and agarose gel electrophoresis for a) PTHrP and b) SOX9.

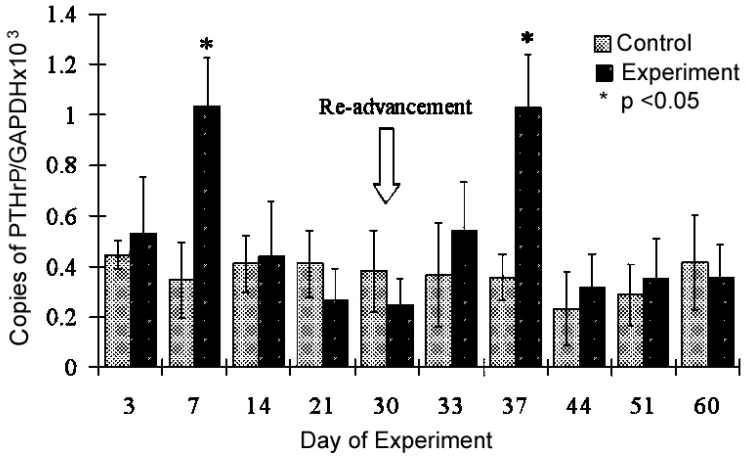

Figure 3. Real-time RT-PCR of PTHrP mRNA expression. The mean values of normalized PTHrP mRNA expression normalized with GAPDH and the standard deviations are shown.

94ºC for 2 minutes, followed by 45 cycles of 94ºC denaturation for 30 seconds, 60ºC annealing for 30 seconds and 72ºC elongation for 45 seconds. To confirm the specificity of the reaction, melting point analysis was carried out by increasing the PCR product temperature from 55ºC to 98ºC at 0.5ºC intervals for every 10 seconds (Figure 2). The quantification of gene expression was done by iCycler iQ™ Real-Time PCR Detection System Software (Bio-Rad). The target gene copy numbers were normalized with the copy numbers of GAPDH.

Primers for amplification were based on published sequences (Wang and Seed, 2003). For SOX9, the sequences used were: sense primer, 5'-GAGCCGGATCTGAAGA-AGGA-3'; antisense primer, 5'-GCTTGACGTGTG-GCTTGTTC-3' (151 bp, GenBank accession number AB073720). For PTHrP, the sequences used were: sense primer, 5'-CACCAGCTACTGCATGACAAGG-3'; antisense primer, 5'-GGTGGTTTTTGGTGTTGGGAG-3' (154 bp, GenBank accession number M31603). For GAPDH, the sequences used were: sense primer, 5'-ATGTTCCAGTAT-GACTCTACCC-3'; antisense primer, 5'-AGCATCACCC-CATTTGATGT-3' (136 bp, GenBank accession number XM237330).

3.4 Statistical Analysis

The data was processed with SPSS for Windows Release 11.0.0 (SPSS Inc.). The target gene expression level was presented as mean copy numbers and standard deviation for each group. To compare the differences in target gene expression level between the groups, 2 sample t-test was performed for each experimental day. The association between the gene expression of SOX9 mRNA and PTHrP mRNA was estimated by Pearson's correlation coefficient.

4. RESULTS

4.1 PTHrP gene expression

In the control group, the target gene was expressed constantly throughout the experiment (Figure 3). The range of mean copies of PTHrP/GAPDH was from

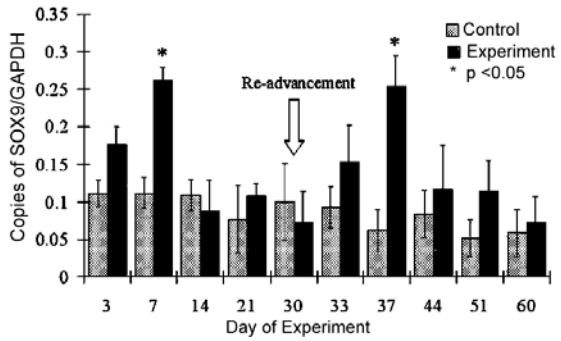

Figure 4. Real-time RT-PCR of SOX9 mRNA expression. The mean values of SOX9 mRNA expression normalized with GAPDH and the standard deviations are shown.

0.231×10^{-3} to 0.445×10^{-3}. While in the experimental group, the PTHrP gene expression was 0.530×10^{-3} at day 3, which was 1.4 fold more when compared with the control. At day 7, the mean PTHrP/GAPDH copies was 1.033×10^{-3}, which was 3.0 fold more when compared with the control group and this difference was statistically significant ($p<0.05$). Afterward the PTHrP level in the experimental group went down to a level similar to that of the control. Upon the second mechanical strain application in the experimental group, the PTHrP expression increased to 0.545×10^{-3}, which was 1.5 fold more when compared with the control at day 33. At day 37, the copies of PTHrP/GAPDH in the experimental group increased to 1.031×10^{-3}, which was statistical significantly (2.9 fold) more than that of control group ($p<0.05$). Following that the PTHrP gene level returned to the level similar to that of the control group.

4.2 SOX9 gene expression

The pattern of the SOX9 gene expression in the control group was similar to that of the PTHrP (Figure 4), the mean copies of SOX9/GAPDH was ranged from 0.052 to 0.112. While in the experimental group, the mean SOX9/GAPDH copies number was 0.177 at day 3, which was 1.6 fold more than that of the control. Then the SOX9 gene expression increased significantly at day 7 to a level of 0.262, which was 2.3 fold more than that of the control ($p<0.05$). Afterward the SOX9 gene expression went down to that of the control until 3 days after second application of mechanical strain. At day 33, the SOX9 expression increased again to a level of 0.153, which was 1.7 fold increase to that of the control. Later the SOX9 level reached 0.253 at day 37, which was significantly more than that of the control group (4.1 fold increase, $p<0.05$). After that the SOX9 expression returned to a level that similar to the control group.

5. DISCUSSION

Results of the current study demonstrated that both PTHrP and SOX9 were strongly related with the degree of mechanical strain applied. In the present study, we quantitatively assessed their pattern of expression following repeated mechanical tensile force application through advancing the mandible in stepwise manner. Such mechanical force led to a significant increase in the level of expression of PTHrP mRNA and SOX9 mRNA in the mandibular condylar cartilage (Figure 3, 4). Recently, we reported that similar tensile mechanical force application led to a significant increase in the expression of Ihh by condylar chondrocytes (24). The direct target of Ihh signaling was the perichondrium, where it induced the expression of a second signal, PTHrP, in the periarticular chondrocytes and regulated the rate of hypertrophic differentiation (25). Results of the current study demonstrated a similar route of action where the first advancement led to a significant increase in PTHrP mRNA expression on day 3 of force application, 1.4 fold increase when compared to control, and reached a peak (3 fold increase) on day 7 of advancement (Figure 3). The expression of PTHrP corresponded to the expression of Ihh which reached a peak on day 7 of advancement (24). Most interestingly, the second advancement which led to a repeated mechanical tensile force application in the condyle, caused a significant increase in PTHrP mRNA expression (1.5 fold), 3 days after the second advancement and reached a similar peak 7 days after the second advancement. This pattern corresponded to the pattern of expression of Ihh under similar conditions with a 12.5 fold increase in peak level (26). These results pointed out that Ihh/PTHrP work in concert, within mandibular condylar cartilage, to regulate the pace of chondrocyte differentiation.

In a recent publication, we reported that increased PTHrP expression as a result of single advancement was associated with the increase of new chondrocyte population (21). It was documented that PTHrP up-regulated SOX9 transcription (22), which had been shown to promote the differentiation of mesenchymal cells into chondroblasts in mandibular condylar cartilage (23). Therefore, in the present study we quantitatively assessed the pattern of expression of SOX9 mRNA and correlated it to PTHrP mRNA expression to improve our understanding of tissue responses to repeated mechanical force application. The first bout of mechanical tensile force application resulted in a significant increase (1.6 fold) on day 3 and reached a peak (2.3 fold) on day 7. The second advancement led to a 1.7 fold increase and 4.1 fold increase 7 days following the second advancement (Figure 4). Showing a very close correlation between SOX9 and PTHrP expression, Pearson correlation coefficient was found to be 0.837 ($p<0.01$), indicated that they are positively related to the degree of mechanical strain applied. Such a significant increase in PTHrP as a result of mechanical force application will lead to a delay in chondrocyte maturation and thus allow further proliferation and replication of the chondrogenic precursors. The increase in population size of replicating mesenchymal cells coincided with a significant increase in SOX9 mRNA expression, which could then act upon these replicating mesenchymal cells and induce their differentiation into chondrogenic cells. The more cartilage cells, the more cartilage matrix to be synthesized (27) and as such it offers a template onto which bone will form. Thus the more cartilage formed as a result of repeated mechanical tensile force application, the more bone that could be laid down during condylar growth. Such a pattern was evident in our earlier work where stepwise mandibular

advancement resulted in a significantly more replicating cells in the condyle, significantly more bone and subsequently more growth (19).

Therefore, in conclusions, repeated force application on the condyle triggers the expression of PTHrP which delays cellular maturation and thus allows proliferating mesenchymal cells to reach their full replicating potential. PTHrP also up-regulates the expression of SOX9, which in turn acts upon the mesenchymal cells, and induces their differentiation into chondrogenic cells.

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