Original Article

Regulation of the Response of the Adult Rat Condyle to Intermaxillary Asymmetric Force by the RANKL-OPG System

Yue Xu[a]; Tuojiang Wu[b]; Yangxi Chen[c]; Zhiguang Zhang[d]

ABSTRACT

Objective: To test the hypothesis that the RANKL-OPG system in the subchondral bone of adult rat condyles does not vary in response to different values of intermaxillary asymmetrical forces.

Materials and Methods: The mandibular rami of 160 Sprague-Dawley rats (3 months old) were subjected to unilateral traction in the anterior-superior direction using an elastic force. We used 120 *g* and 40 *g* as the initial elastic forces, and then removed the traction after 28 days. The expression of RANKL and OPG in the subchondral bone of the condyles was analyzed by semi-quantitative immunohistochemistry.

Results: Different force levels induced similar changes in the expression of the OPG protein by 28 days. However, the effect of a 120-*g* elastic force on the expression of RANKL was stronger than that of a 40-*g* force. Because of the asynchrony of RANKL responses to external forces of different values, the values of RANKL/OPG ratio showed characteristic variation. The RANKL/OPG ratio in the side treated with heavy force showed a distinct negative correlation with the value obtained when a light force was used.

Conclusions: The hypothesis is rejected. Different values or traction force cause a variation of the RANKL/OPG ratio through the expression of RANKL protein, modulating the activities of bone remodeling in the subchondral bone of condyle. (*Angle Orthod.* 2009;79:646–651.)

KEY WORDS: Traction; Temporomandibular joint; Osteoclasts; RANKL; Osteoprotegerin; Bone turnover marker

INTRODUCTION

The causal linkage between mandibular displacement and changes in the growth of the mandible has been debated for decades. A considerable body of evidence derived from animal studies attests to the likelihood that growth of the condyle can be altered after a change in the postural position of the mandible.[1–2] In growing rats, a lateral functional shift of the mandible resulted in increased thickness of the condylar cartilage that was followed temporally by increased proliferation of prechondroblastic cells on the protruding side. Additionally, the condylar shape, size, and trabecular bone pattern appear to respond to changes in loading of the temporomandibular joint (TMJ).[3–4]

Currently, the manner in which an asymmetric force affects an already mature TMJ is unknown. Due to its low capacity for compensation, remodeling of the adult TMJ is slow and under great constraint, and the TMJ becomes more sensitive to elements such as counter-stress. The incidence of temporomandibular disorders (TMDs) in the normal population is 40%–60%, and these affect mostly young adults.[5] Remodeling, degeneration, and osteoarthropathy of the adult TMJ often occur simultaneously, but the mechanisms by which these occur are not well-characterized.

The adult TMJ retains the ability to adapt to alterations in the mechanical equilibrium of the joint, regardless of the cessation of skeletal growth.[6] However, only 1% to 3% of load forces are attenuated by the

[a] Lecturer, Department of Orthodontics, Guanghua School of Stomatology, Sun Yat-sen University, Guangzhou, Guangdong, China.

[b] Lecturer, Department of Orthodontics, the Affiliated Hospital of Medical School, Nanjing University, Nanjing, Jiangsu, China.

[c] Professor, Department of Orthodontics, West China College of Stomatology, Sichuan University, Chengdu, Sichuang, China.

[d] Professor, Department of Oral and Maxillofacial Surgery, Guanghua School of Stomatology, SUN Yat-sen University, Guangzhou, Guangdong, China.

Corresponding author: Dr Yangxi Chen, Department of Orthodontics, West China College of Stomatology, Sichuan University, No. 14 The South of Road People, Chengdu, Sichuang 610041, China
(e-mail: cyxlfx@gmail.com)

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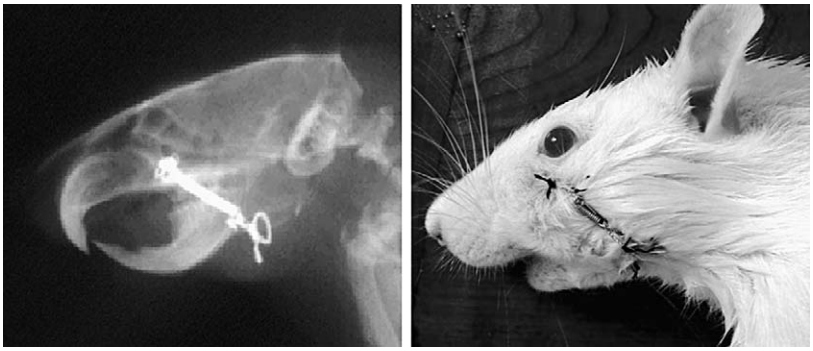

Figure 1. Animal model. Cephalogram of the animal model (left). A nickel-titanium (Ni-Ti) coil spring was placed between the left mandibular angle and anterior left zygomatic arch of the rats (right).

cartilage layer of the condyle. The subchondral bone is a better shock absorber and protects the hyaline cartilage against damage caused by excessive loads.[7] The role of subchondral bone in the etiology and progression of cartilage disease has likely been underestimated, although the earliest scientific reports about its role in disease appeared in the 1920s.

Bone remodeling is one of the main metabolic activities necessary for maintaining the normal structure and function of bones. Osteoclasts play an important role in these processes; osteoprotegerin (OPG), receptor activator of NF-κB (RANK), and RANK ligand (RANKL) coregulate the functions of osteoclasts.[8] A wide range of cytokines reportedly influence bone resorption through the RANKL/OPG axis, which is perhaps best viewed as a final, convergent step in the promotion of osteoclastogenesis and osteoclast activity for the upstream resorptive cytokines.[9] The proportion of RANKL and OPG protein (RANKL/OPG ratio) in the local bone microenvironment determines the direction of differentiation of osteoclasts.

In the present study, we investigated the expression of RANKL and OPG in the condyle in an animal model in which the mandibular position was shifted by quantitative asymmetric traction. The mechanisms by which the traction forces resulted in differential modulation of regulation of the osteoclasts in the subchondral bone of the condyles were also investigated.

MATERIALS AND METHODS

One hundred eighty 10-week-old male Sprague-Dawley (SD) rats, 350 ± 50 g, were used in the experiment. One hundred sixty SD rats were randomly divided into a light force group that was subjected to a traction force of 40 *g* and a heavy force group subjected to a traction force of 120 *g.* The rest were used as a control.

General anesthesia was induced by an intraperitoneal injection of pentobarbital (50 mg/kg body weight). An incision was made parallel to the left eyelid at approximately 4 mm to the anterior-inferior orbit to expose the zygomatic arch and the zygomatic process of the superior maxillary bone. Another notch was cut near the left mandibular angle, 1 cm ventral of the underlip and 5 mm dextral to the midline of the belly. A nickel-titanium coil spring was fixed between the left mandibular angle and the anterior left zygomatic arch by using steel stainless wires. The force was measured by a dynamometer and the traction forces used in this experiment were 40 *g* and 120 *g* (Figure 1). The incisions were sutured after surgery. Some of the rats (10/group) were sacrificed 3, 7, 14, or 28 days after the application of traction. In all other animals, the coil springs were removed 28 days after the traction was applied. The animals were sacrificed at 3, 7, 14, or 28 days after the removal of traction. The control rats were subjected to the same surgery to expose the zygomatic arch and the mandibular angle, but no spring was attached.

After the experimental period, the complete TMJs were resected to determine the expression of OPG and RANKL by immunohistochemistry assay. The primary antibodies against OPG and RANKL were purchased from Santa Cruz Biotechnology, Heidelberg, Germany. In the negative controls, the primary antibody was replaced with phosphate-buffered saline (PBS).[10] The positive cells and the distribution of positively stained tissues were analyzed under a microscope at a high-power magnitude (200×). The subchondral bone was processed as the area of interest. The positive signal intensity (integrated optical density, IOD) of the subchondral bone of the condyle was used as the parameter for semiquantitative determination, and measured with the assistance of a computer-based Image-Pro Plus program (Media Cybernetics, Bethesda, Md). All readings were done independently by two blinded observers, and the mean values were taken as the final results.

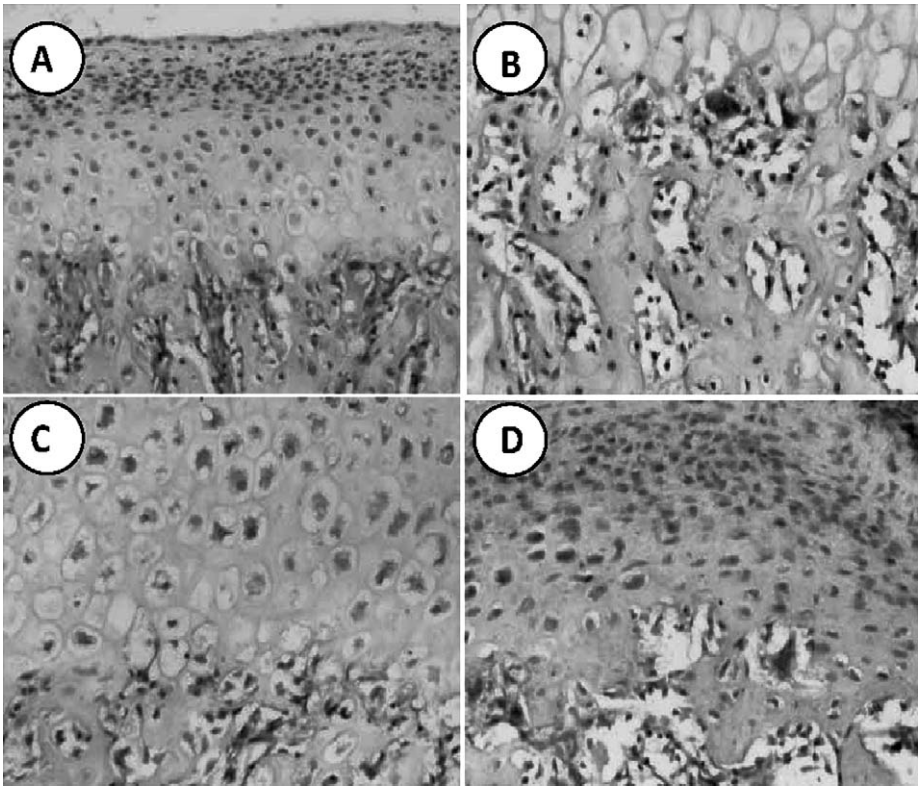

Figure 2. OPG and RANKL protein expression in adult condyle. Sections were immunostained with 1:50 diluted OPG (A, B) and 1:25 diluted RANKL (C, D) antibodies. (A) The layer and location of OPG expression in the condyle, 100×. (B) OPG in the subchondral bone of the condyle, 200×. (C) RANKL expression in the chondrocytes of the condyle, 200×. (D) RANKL expression in the subchondral bone of the condyle, 200×.

Statistical Analysis

Statistical analyses were processed in SPSS 11.5 software, (SPSS Inc, Chicago, IL) including the comparison of the values of RANKL/OPG expression in the subchondral bone and correlation analysis for the curves obtained from the light force and heavy force groups and for the control and treatment sides of the animals. The differences between the groups were analyzed using nonpaired Student's *t*-test. A *P* value less than .01 was considered statistically significant.

RESULTS

The expression of OPG and RANKL protein was examined in adult rat condyles. OPG was expressed throughout the condyle cartilage, and was strongly expressed in the chondrocytes and the hypertrophic zones (Figure 2A). RANKL was expressed throughout the chondric lamina of the condyle, and was highly expressed in the maturation zone of chondrocytes. The hypertrophic chondrocytes often showed cytoplasmic labeling (Figure 2C).

In the subchondral bone, the osteoblasts and osteoclasts were also intensely positive for OPG and RANKL staining. The IOD value for OPG in the subchondral bone of the condyles in the control group was 23.78 ± 1.42, whereas the IOD for RANKL was 5.89 ± 0.70. Therefore, the RANKL/OPG ratio in the control group was 0.246 ± 0.013. The detailed data and results of statistical analyses are shown in Tables 1 through 3 and in Figures 3 and 4.

The expression of RANKL in the side subjected to the light force increased dramatically within the first 3 days (Table 2, $P < .01$), returned to its normal level by day 7, but demonstrated another peak on day 14

Table 1. OPG Expression in the Subchondral Bone of the Condyle Subjected to Force[a]

Time/Group, Days	Light Force, *g*	Heavy Force, *g*
Control Group	23.78 ± 1.42	23.78 ± 1.42
3	23.18 ± 1.58	21.52 ± 1.16*
7	9.14 ± 1.22*	8.34 ± 0.93*
14	24.33 ± 1.32	26.11 ± 1.45*
28	23.24 ± 1.60	25.30 ± 0.90*
31	9.77 ± 0.84*	15.28 ± 0.63*
35	24.19 ± 1.21	24.66 ± 1.16
42	16.77 ± 0.86*	12.47 ± 0.60*
56	18.30 ± 1.48*	20.24 ± 1.24*

[a] The positive signal intensity (integrated optical density, IOD) of OPG protein in the subchondral bone of the condyle was measured as described in the Methods section. Values are expressed as mean ± SEM. The data were compared using nonpaired Student's *t*-test.
* $P < .01$ compared with the control group.

Table 2. RANKL expression in the Subchondral Bone of the Condyle Subjected to Force[a]

Time/Group, Days	Light Force, *g*	Heavy Force, *g*
Control Group	5.89 ± 0.70	5.89 ± 0.70
3	13.81 ± 1.79*	22.67 ± 1.33*
7	6.28 ± 1.13	7.66 ± 0.79
14	11.71 ± 0.87*	14.67 ± 1.59*
28	7.37 ± 0.89*	34.10 ± 1.29*
31	12.54 ± 1.75*	7.06 ± 0.53*
35	3.62 ± 0.22*	24.98 ± 0.96*
42	2.42 ± 0.21*	8.56 ± 0.60*
56	6.17 ± 0.78	14.30 ± 0.94*

[a] The positive signal intensity (integrated optical density, IOD) of RANKL protein in the subchondral bone of the condyle was measured in the experiment. Values are expressed as mean ± SEM. The data were compared using nonpaired Student's *t*-test.

* $P < .01$ compared with the control group.

(Table 2, $P < .01$). The removal of the external force created a new imbalance. A second peak occurred between day 3 and day 7 (Table 2, $P < .01$) after the removal of traction, and the IOD declined again by day 14. The effect of a 120-*g* elastic force on the expression of RANKL was stronger than that of a 40-*g* force (Figure 3, $P < .01$) except on day 7.

Different force levels can induce similar changes in the expression of the OPG protein by 28 days (Figure 3, $P > .01$). The expression of OPG in the side subjected to forces varied minutely within the first 3 days, decreased at day 7 (Table 1, $P < .01$), and reached the first peak by day 14; after the removal of traction, the IOD declined a second time by day 3 (Table 1, $P < .01$) and then increased until it reached another peak on day 7. The strength of the force (heavy/light) had no noticeable effect on OPG expression (Figure 3).

Correlation analysis was performed to compare the ratios of RANKL/OPG between the heavy force group and the light force group (Table 3). The coefficient for the groups was 0.005. The concomitant probability for the statistical test was 0.99. Therefore, the ratios of RANKL/OPG for the groups may be considered to have no correlation.

Table 3. RANKL/OPG Ratio in the Subchondral Region of the Mandibular Condyle[a]

Time/Group, Days	HT	LT
Control Group	0.246 ± 0.013	0.246 ± 0.013
3	1.053 ± 0.071	0.596 ± 0.037
7	0.918 ± 0.061	0.687 ± 0.034
14	0.562 ± 0.067	0.483 ± 0.021
28	1.348 ± 0.041	0.317 ± 0.022
31	0.462 ± 0.041	1.284 ± 0.089
35	1.013 ± 0.064	0.150 ± 0.004
42	0.686 ± 0.039	0.144 ± 0.012
56	0.707 ± 0.026	0.337 ± 0.018

[a] Values are expressed as mean ± SEM. HT: the side that was exposed to heavy force (120 *g*); LT: the side that was exposed to light force (40 *g*).

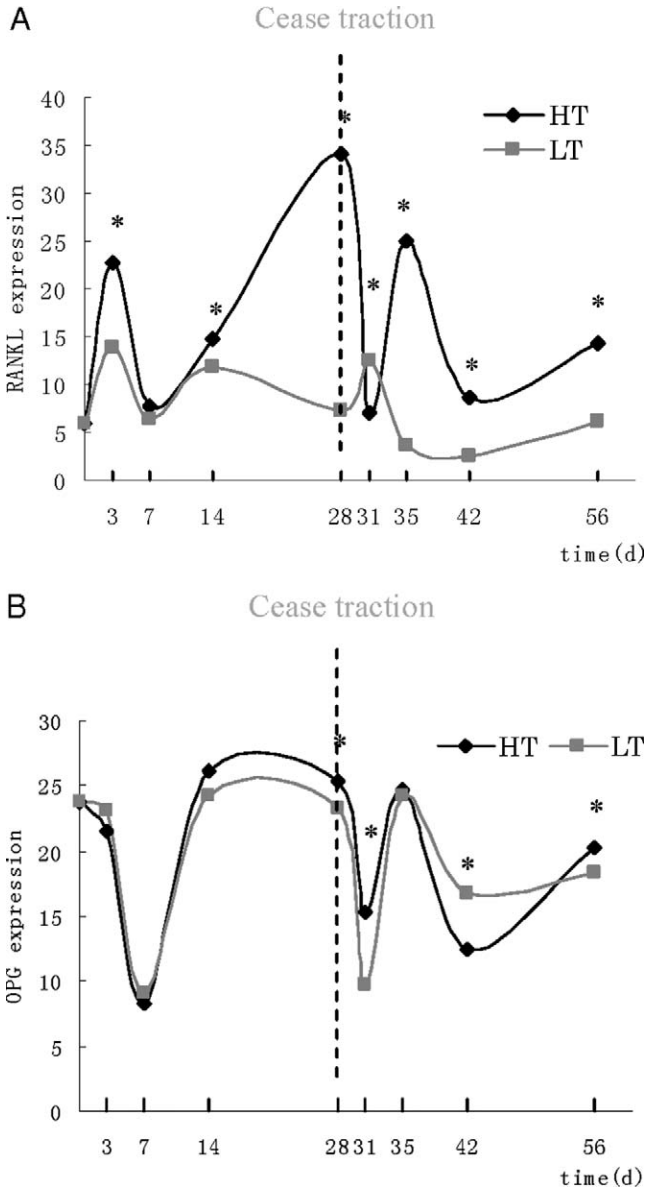

Figure 3. Integrated optical density (IOD) values of RANKL and OPG expression in the subchondral bone of the mandibular condyle in the sides exposed to the force. The dotted line indicates that the coil springs between the left mandibular angle and anterior left zygomatic arch of the rats were removed 28 days after the traction was applied. The data were analyzed using nonpaired Student's *t*-test. HT: the side that was exposed to heavy force (120 *g*); LT: the side that was exposed to light force (40 *g*).* $P < .01$ ht vs lt.

DISCUSSION

Intermaxillary asymmetrical traction, a method routinely used in orthodontics to correct mandibular position and adjust occluding relation, is conventionally employed as compensation treatment for adult jaw deformity. The treatment presumably affects the forces

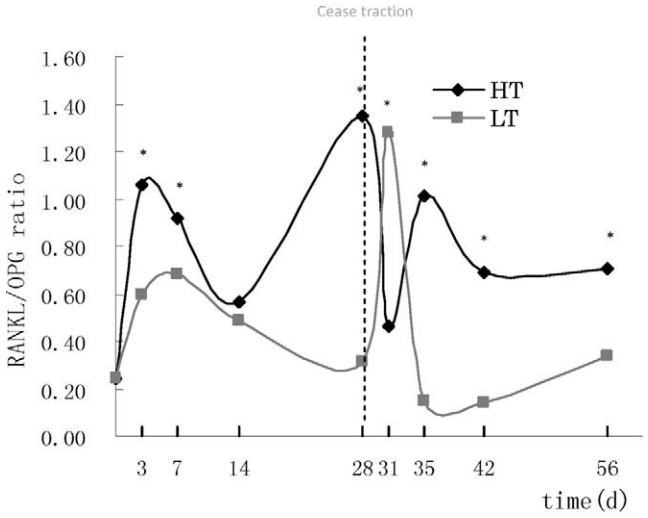

Figure 4. The RANKL/OPG ratio in the subchondral bone of the condyle exposed to the force. The dotted line meant that the coil springs between the left mandibular angle and anterior left zygomatic arch of the rats were removed 28 days after the traction was applied. The data were analyzed using nonpaired Student's *t*-test. HT: the side that was exposed to heavy force (120 *g*); LT: the side that was exposed to light force (40 *g*). * $P < .01$ ht vs lt.

developed on the dentoalveolar region, the mandibular and maxillary bones, and the two TMJs. In our study, the asymmetric traction forced the left condyle to become positioned anteriorly (protruded) relative to normal.

Once formed, subchondral bone undergoes a remodeling process that involves breakdown (resorption) and formation (synthesis) of bone. Cell-cell contact between osteoclasts and osteoblasts and signaling through the RANKL/RANK/OPG pathway play an important role in the regulation of bone remodeling.[11] These three major proteins (RANKL, RANK, and OPG) have been shown to be the key players in regulating bone remodeling. RANKL, expressed by osteoblasts, directly activates cells of osteoclast lineage by binding to the membrane receptor RANK. OPG, which is mainly produced by cells of the osteoblast lineage, but can also be produced by the other cells in the bone marrow, acts as a decoy receptor for RANKL. In our study, we have shown that OPG and RANKL were expressed to a high degree by mesenchymal cells, proliferative chondrocytes, osteoblasts, and osteoclasts. Our results are in line with those of other studies concerning RANKL and OPG protein localization in the femur of growing rats,[11] or in developing TMJs in humans.[12]

Our study elucidated the changes in RANKL protein expression that occur in bone lineage cells during exposure to traction force. The strength of the force has obvious influence on the expression of RANKL. RANKL can activate mature osteoclasts in a dose-dependent manner in vitro, and can rapidly lead to the resorption of bone in vivo by activating preexisting osteoclasts.[13] RANKL increases the survival of the mature osteoclasts in vitro and in vivo, which may be due to the ability of these factors to induce NF-κB activity.[14] The IOD value of the heavy force group increased constantly at least until day 14, and declined by day 28. In contrast, the expression of RANKL in the light force group began to decline by day 14. Therefore, it appears that the osteoclasts in the condyle had a more intense and enduring response to the 120-*g* force than to the 40-*g* force.

When an external force was exerted on the mandible, bone remodeling of the subchondral bone of condyle began immediately, as evidenced by our experimental results. The appearance of the first peak in the IOD curve indicated the activation of the osteoclasts. The lifespan of an osteoclast is only about 12 days, and progressive remodeling must be sustained by the continued arrival of mononuclear osteoclast precursors to replace the osteoclasts lost due to apoptosis. When the supply of osteoclast precursors is exhausted, the "basic metabolizing units" of bone reach the end of their lifespan. Recently, remodeling activation was redefined as the conversion of a region of bone surface from quiescence to active remodeling, a process requiring local recruitment of new osteoclasts. Our data indicated that the expression of RANKL reached a second peak at day 14, paralleling the differentiation of new stromal cells to osteoclasts in order to continue the remodeling process and form new bone. The ratio of RANKL/OPG after the removal of traction showed a similar variation.

The general function of OPG is to regulate bone resorption by inhibiting terminal differentiation and activation of osteoclasts and inducing apoptosis of osteoclasts.[15] In our study, the IOD of the side subjected to force varied minutely within the first 3 days and decreased at day 7. The strength of the force (heavy/light) had no noticeable effect on OPG expression. Hence, within the first 3 days of applying the traction force, the metabolism of the condyle was enhanced, subchondral bone remodeling was initiated, and most osteoclasts were activated; this resulted in negative feedback on OPG expression. As bone absorption and the osteogenetic process gradually reached a new balance by 2 weeks, the metabolic activity was enhanced as a whole, and the OPG level reached its first peak. The removal of the external force created a new imbalance. The level of OPG expression was similar to that in the previous process, fluctuating from low to high as the time after the imbalance elapsed.

We believe that our study demonstrates that OPG expression in the subchondral bone has no measurable difference in response to light and heavy traction

and varies synchronously. Further, while there were no major differences in the pattern of RANKL responses to different values of traction, the initiation and duration of the peaks in expression in response to light traction and heavy traction were asynchronous. The data suggest that this asynchronous mechanism reflects the intensity of the remodeling in the condyle.

Given the asynchrony of the responses of RANKL to external forces of different magnitudes, it is not surprising that the ratios of RANKL/OPG showed characteristic variations. The ratio of RANKL/OPG, in fact, may be the ultimate determinant of bone resorption. In the first 7 days, bone resorption is stimulated by both increased RANKL and decreased OPG, which can amplify proresorptive signals. Our results are in line with those of other studies concerning the RANKL/OPG ratio in primary hyperparathyroidism.[16] These changes were closely followed by increases in osteoclast numbers and serum calcium levels.[17] The RANKL/OPG values in both of the groups reached their first peaks by the first week. The ratio in the group exposed to the heavy force reached a second peak by 4 weeks, which was 7-fold higher than the normal value. In experimental zoology, the period for bone metabolism in humans is 2.5- to 5-fold that in rats; therefore, the above results suggest that although functional force traction was performed in orthodontic treatments, joint remodeling lagged behind jaw transformation. To investigate whether irreversible pathologic changes occurred, it would be necessary to continue observation for at least 2 months after cessation of traction.

CONCLUSIONS

- The remodeling of the subchondral bone of the adult TMJ could be activated by external forces. Different force levels can induce similar changes in the expression of the OPG protein.
- Different values of intermaxillary traction force not only influence the curve shape of the RANKL protein expression, but change the expression level of RANKL protein in the subchondral bone.
- Different values of intermaxillary traction force regulate the activity level of the remodeling in the adult condyle by modulating the expression of RANKL in the RANKL-OPG system.

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