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Subchondral bone loss following orthodontically induced cartilage degradation in the mandibular condyles of rats

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ABSTRACT

Osteoarthritis (OA) is a degenerative joint disease generally characterized by progressive cartilage degradation and subchondral bone changes. Subchondral bone changes have been proposed to initiate or accompany with cartilage degradation in OA. The purpose of this study was to characterize cartilage damage, subchondral bone remodeling, and the possible mechanism involved in these morphological changes in our reported rat model with OA-like lesions in the mandibular condyle.

In experimental groups, the dental occlusion was orthodontically disturbed. By histological analysis, transmission electron microscopy (TEM), micro-CT scanning and serum tests, changes in condylar cartilage and subchondral bone were analyzed at 8 and 12 weeks after treatment. The mRNA and protein levels of bone pro-resorptive and pro-formative factors by chondrocytes were investigated. Increased degraded cartilage areas and obvious cartilage calcification were observed in 8- and 12-week treated (EXP) groups compared to the age-matched controls. Subchondral bone loss, characterized as decreased bone mineral density (BMD), bone volume fraction (BV/TV) and trabecular thickness (Tb.Th), but increased trabecular separation (Tb.Sp), was observed in the 12-week but not the 8-week EXP group, respectively, versus their age-matched controls. The subchondral bone loss in the 12-week EXP group was accompanied with decreased new bone formation rate, but increased serum carboxy terminal telopeptides (CTXs), and increased osteoclast numbers and proportion of surface area in the subchondral bone regions. Increased mRNA and protein levels of M-CSF, VEGF, RUNX and RANKL/OPG ratio, but decreased OPG, were found in condylar cartilage in the 12-week EXP group versus its age-matched controls, and those of RANKL/OPG ratios were significantly higher in the 12-week EXP group than the 8-week EXP. In addition, increased mRNA levels of VEGF, RUNX and RANKL/OPG ratio, but decreased OPG, were also found in condylar cartilage in the 8-week EXP group versus its age-matched controls (All $P<0.05$).

This study demonstrated that obvious subchondral bone loss followed cartilage degradation in the mandibular condyles in the present rat models and suggested that the imbalance of chondrocyte-secreted regulatory factors within the degraded cartilage may play a role in the osteoclastogenesis, and thus leading to the subchondral bone loss in OA.

Introduction

Osteoarthritis (OA) is characterized by a slow, progressive degradation of articular cartilage and changes in the subchondral bone [1]. Although the initiation and progression of OA involve a disruption of an equilibrium between cartilage and subchondral bone, the sequence in which cartilage degradation and subchondral bone changes occur is still controversial [2]. Some studies reported that subchondral bone changes happen simultaneously or following cartilage degradation [2–4], whereas others argued that subchondral bone changes precedes the cartilage degradation and thus play important roles in cartilage degradation [5–7].

The temporomandibular joint (TMJ) is one of the most common sites of occurrence of OA [8,9]. A number of experimental methods for producing TMJ OA models have been described, including surgical manipulation of the joint structure [10], local application of chemicals [11], excessive length or frequency of mouth opening [12], and genetic modification in animals [13]. Because the functional movement and biomechanical conditions of TMJ are closely related to dental occlusion, and abnormalities in dental occlusion are considered as one of the potential etiological factors for TMJ OA [14], we recently described the ability to create OA-like lesions in TMJ cartilage of rats

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by a specially designed abnormal occlusion that highly simulated clinical dental occlusal problems in humans [15,16]. Thus this animal model is believed to be suited for the study of TMJ OA.

Subchondral bone remodeling in OA, involving both bone resorption and formation, has been extensively studied [17]. Some reports have indicated that, in the late stage of OA, increased trabecular bone volume fraction and trabecular thickness was predominant in human knee OA samples [18,19], and in knee OA animal models [20]. Conversely, other studies focusing on the early phase of the disease have, in general, identified decreased trabecular bone volume fraction and trabecular thickness, as well as a decrease in bone mineral density (BMD) in human knee OA specimens [7,21], and in some knee OA animal models [6,22]. The bone losses in these OA samples are reported to be associated with the process of osteoclastogenesis [22,23], during which the receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) act as the final effectors [24]. An increase in RANKL/OPG ratio in the local microenvironment could contribute to the excess osteoclastogenesis, and in turn lead to the osteoclastic bone resorption in OA [25,26]. Macrophage-colony stimulating factor (M-CSF) is also required for the osteoclast differentiation and activation [24]. The presence of M-CSF and RANKL was shown to be sufficient for osteoclast differentiation from spleen cells *in vitro* [27]. The detailed biological effects of RANKL/OPG ratio and M-CSF on the bone resorptive process have been intensively investigated within the bone tissue, osteoblasts and osteoclasts [24]. Interestingly, chondrocytes were reported to express these pro-resorptive factors. OPG and RANKL have been detected in the superficial zone of normal cartilage, whereas during OA their expression was found to extend to the middle zone [28]. It was recently reported that the expressions of M-CSF and RANKL/OPG ratio are significantly increased in the OA knee chondrocytes, respectively, in the monoiodoacetic acid induced OA animal model [29] and in OA patients [30]. Thus the roles of these pro-resorptive factors secreted by the chondrocytes in the onset and progression of subchondral bone changes in OA are suggested but remain to be confirmed.

In physiologic conditions, the activity of the bone resorption is well coupled with that of the bone formation such that bone mass is maintained [31]. In the TMJ condyle, the subchondral bone is mainly formed by endochondral ossification which is controlled by factors that are endogenously expressed by chondrocytes [32]. Among them, RUNX2 mediates chondrocyte terminal maturation and promotes endochondral ossification [33], and VEGF regulates the replacement of cartilage by bone and thus is essential for this process [34].

In the present study, we used our recently reported rat model with obvious cartilage degradation in the mandibular condyle to test the onset sequence of the changes in the subchondral bone and the degradation in cartilage by morphological investigation of the mandibular condyle. We further evaluated the mRNA and protein levels of bone pro-resorptive factors (M-CSF, RANKL and OPG) and bone pro-formative factors (Runx2 and VEGF) in the mandibular condylar cartilage of these rats.

Materials and methods

Animal experiments

Eighty-four female Sprague–Dawley rats (weight 180-190 g) at 8 weeks of age were provided by the animal center of the Fourth Military Medical University. All procedures and the care administered to the animals were approved by the University Ethics Committee, and performed according to institutional guidelines. In the experimental groups (EXP groups), disordered occlusion was created as we previously described [15]. In the sham-treated groups (control groups), rats received the same procedure, but the dental occlusion was not disturbed. Tetracycline (25 mg/kg; Amresco Ltd., USA) and calcein (5 mg/kg; Sigma Chemicals Co., USA) labels were respectively injected in 6 out of 21 rats in each group at 14 and 4 days before sacrifice. No rat showed any sign of disability, and they all received the same standardized diet throughout the procedure.

Sample preparation and experimental procedure

Animals were sacrificed at the end of the 8th or 12th week after the beginning of the experiment. The TMJ tissue blocks of the 6 fluorescently labeled rats were embedded in methylmethacrylate. The right condyles were stained by H&E for evaluation of the bone structural parameters, while the left ones were used for the measurement of dynamic new bone formation. The sera of the other 15 unlabeled rats were collected for the measurement of bone turnover markers. For 6 out of the 15 unlabeled rats, the right condyles were decalcified and embedded in paraffin wax for histochemical and immunohistochemical staining. The left condyles used for micro-CT scanning (GE eXplore Locus SP, London) were separated from the mandibular skulls [35], and were immediately fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 h at 4 °C. After micro-CT scanning, the cartilages were dissected under a dissecting microscope (SZX9, Olympus, Japan) and were observed by transmission electron microscopy (TEM). The total cartilage from the other 9 unlabeled rats were dissected and used for real-time polymerase chain reaction (PCR) analysis.

Bone histomorphometry

As shown in Fig. 1a, two square frames (each 0.5 mm × 0.5 mm) under the osteochondral interface were located at the middle of the center and posterior thirds of the mandibular condyle. Within the selected squares, bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp) were calculated [35].

The unstained sections with sequential labeling were firstly observed under a fluorescence microscope (BX-60, Olympus, Japan), and then observed by a confocal microscopic system (FV1000, Olympus, Japan) to study the dynamics of osteogenesis [36]. Briefly, the desired fluorescent signal was obtained with a DAPI filter for tetracycline (excitation 405 nm; emission 470–480 nm) and Alexa Fluor filter for calcein (excitation 488 nm; emission 510–520 nm). Red pseudo color was applied to the tetracycline fluorescent image to obviously distinguish from the green images of calcein. After image acquisition, the fluorescent areas were measured within above selected frames under the osteochondral interface (Fig. 1a). The temporal new bone formation were calculated and interpreted as the ratio of those areas labeled with calcein (green) and tetracycline (red) (Fig. 1c–h). This method was previously used in the dynamic measurement of "chaotic" osteogenesis in fracture healing [36], and was adopted in the present study because in TMJ condyle the subchondral bone near to the osteochondral interface was irregular with substantial imaging overlap of the fluorescent labeling.

Histochemical and immuohistochemical staining

H&E and toluidine blue staining were used for histological assessment [15]. Tartrate-Resistant Acid Phosphatase (TRAP) staining was used for the identification of osteoclasts following the manufacturer's instructions (Sigma 387-A, St Louis, USA). A standard, three-step, avidin-biotin complex immunohistochemical staining protocol was carried out [15]. The primary antibodies were anti-OPG (sc-8468; 1:75 dilution), anti-RANKL (sc-7628; 1:75 dilution), anti-M-CSF (sc-1324; 1:75 dilution), anti-VEGF (sc-7269; 1:100 dilution) and anti-RUNX2 (sc-8566; 1:100 dilution), all commercially available from Santa Cruz Biotechnology. In negative control slides, non-immune goat serum was substituted for the primary antibody [15].