# Original Article Subchondral bone changes and chondrogenic capacity of progenitor cells from subchondral bone in the collagenase-induced temporomandibular joints osteoarthritis rabbit model

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Received July 6 2015; Accepted August 21, 2015; Epub September 1, 2015; Published September 15, 2015

Abstract: Purpose: The goals of this study were to characterize subchondral bone changes, and to determine biological activity characteristics of progenitor cell populations from subchondral bone in the collagenase-induced temporomandibular joint osteoarthritis (TMJOA) rabbit model. Greater understanding of such pathological changes occurring in TMJOA samples is critical in the future treatment modalities regarding cartilage protection and repair. Furthermore, the use of progenitor cell populations in various cartilage regeneration strategies proves to be a fruitful avenue for research and clinical applications. Materials and methods: Bone remodeling and anabolic activity of subchondral bone was evaluated by hematoxylin-eosin (H&E), Alcian blue-periodic acid-Schiff (AB-PAS) staining and immuohistochemical staining. The biological activity characteristics of progenitor cells were assessed by expressions of collagen type II, CD44, SOX-9 and MMP-9 by immunohistochemistry and Western blot analysis. Results: In most of the specimens, cartilage of the digested area displayed a reaction characterized by thickening of the cartilage cellular structure with retraction structure formation in the subchondral bone. Most of the specimens focuses on chondroid metaplasia were observed in the subchondral bone, promoting its remodeling, which could develop to endochondral ossification and increasing subchondral bone size. Meanwhile, immunohistochemistry analysis revealed that CD44 expressions in subchondral bone were most significantly increased in TMJOA at 2 weeks group (P < 0.01). And, at 4, 6 and 8 weeks groups, the osteochondral junction had completely disappeared by active subchondral bone remodeling, and collagen type II, CD44, SOX-9 and MMP-9 expressions in active subchondral bone region were significantly increased in TMJOA (P < 0.05). In addition, western blot analysis revealed that CD44 expression significantly emerged in subchondral bone region at 2 weeks group (P < 0.01). Meanwhile, SOX-9 expression emerged in all group, and the intensity was increased in the experimental groups (P < 0.05). Conclusion: Our results suggest that the beneficial activation of progenitor cells and bone marrow stem cells in subchondral bone at early stage of TMJOA played an important role on renovation and remodeling of subchondral bone.

Keywords: Subchondral bone, temporomandibular joint, osteoporosis, progenitor cells

#### Introduction

Osteoarthritis (OA) is the most common musculoskeletal disease worldwide [1], and the temporomandibular joint (TMJ) is one of the most common sites of occurrence of OA [2]. OA is generally considered to be the product of systemic, local, mechanical and metabolic factors having as a result the progressive destruction of joint environment [3]. Common denominators of OA progression are cartilage breakdown, subchondral bone erosion and sclerosis, synovial inflammation and bone marrow edema [4]. Notably, OA is characterized by an imbalance in cartilage and underlying subchondral bone homeostasis [5]. More importantly, subchondral bone changes in OA are potentially both a result and a cause of cartilage loss. Numerous studies have shown that subchondral bone stiffness is able to decrease its viscoelastic properties and produce a loss of shock absorbing capacity, which in turn causes significant mechanical load and breakdown of the overlying cartilage [6]. Consequently, Subchondral bone has become a target of current OA treatments, since subchondral bone changes in OA induce cartilage loss, and cartilage damage may, in turn, have a negative influence on subchondral bone [7].

Highly concerned, progenitor cells of subchondral bone were believed to plays an important role in chondrogenic differentiation and subchondral bone remodeling in the pathogenesis of OA. Evidences exist that osteoarthritic cartilage actually contains increased progenitor populations compared to normal cartilage. Shapiro, F. et al found that human subchondral progenitor cells had cell surface antigens known to be typical of mesenchymal stem and progenitor cells, such as CD73, CD90, CD105, and CD166 [8]. Significantly higher numbers of CD105<sup>+</sup>/CD166<sup>+</sup> multipotential progenitor cells were observed in primary cell cultures derived from human OA compared to those originating from normal cartilage of skeletally mature donors [9]. Therefore, progenitor cells of subchondral bone are capable of differentiating into several different lineages and have been considered as a candidate cell source for cartilage repair and regeneration in OA [10].

Moreover, the microenvironment of OA subchondral bone is likely to have an influence on the ability of stem cells to regenerate articular cartilage, and subchondral bone matrix may respond in a different way to differentiation stimuli due to signaling factors secreted from neighboring OA-chondrocytes or osteoblasts. Several studies reported that progenitor cells of subchondral bone promoted the chondrogenic maker genes such as type II collagen, aggrecan, cartilage link protein, and cartilage oligomeric matrix protein. Despite this, cartilage regeneration is inefficient, and the fibrocartilage resulting from this process is structurally and functionally inadequate [11]. Therefore, the use of local progenitor cell populations in various cartilage regeneration strategies proves to be a fruitful avenue for research and clinical applications.

The goals of this study were to characterize subchondral bone changes, and to determine biological activity characteristics of progenitor cells from subchondral bone in the collagenase-induced temporomandibular joint osteoarthritis (TMJOA) rabbit model. Greater understanding of such pathological changes occurring in TMJOA samples is critical in the future treatment modalities regarding cartilage protection and repair.

# Materials and methods

#### Animal experiments

Male New Zealand white rabbits (2.8-3.0 kg, nine to ten weeks)were obtained from the animal experimental center of Jilin University (Changchun, China) and individually housed with water and food available ad libitum. The bilateral temporomandibular joints (TMJ) of all rabbits were intra-articularly injected with either  $250 \,\mu$ L of 4 mg/mL collagenase solution (Clostridium histolyticum type II, 425 units/mL enzyme activity) or saline (control group).

# Sample preparation and experimental procedures

Per group of five rabbits were sacrificed at the end of the second, 4th, 8th and 10th week after the beginning of the experiment. After removal of the brain, the right TMJs were dissected and fixed in 10% phosphate-buffered formalin for 2 weeks at room temperature, then decalcified in 20% ethylene diamine tetraacetic acid (EDTA) for 3 weeks, and then embedded in paraffin. Standard side sections of 5  $\mu$ m were stained with hematoxylin and eosin (H&E), histochemistry and immunohistochemistry in the cartilage, subchondral bone and bone. The total cartilage from the left unlabeled TMJs were dissected and used for real-time polymerase chain reaction analysis.

# Histochemical and immuohistochemical staining

Hematoxylin-eosin (H&E), Alcian blue-periodic acid-Schiff (AB-PAS) staining was used for histological assessment. The primary antibodies were anti-SOX-9 (1:75 dilution), anti-CD44 (1:75 dilution), anti-collagen type II (1:100 dilution), and anti-MMP-9 (1:100 dilution), all commercially available from Abcam Biotechnology and BIOSS Biotechnology. In negative control slides, non-immune goat serum was substituted for the primary antibody.

#### Western blot analysis

Subchondral bone was obtained from the mandibular condylar of New Zealand white rabbit. Specimens were ground in liquid nitrogen, using a mortar and pestle. Nuclear and cytosolic fractions of tissue lysates were extracted using PRE-PROP lysis buffer. Aliquots of nuclear and cytosolic extracts were separated using sodium dodecyl sulfate-polyacrylamide gel



**Figure 1.** In the experimental groups (A-E), H&E staining in the cartilage and the subchondral bone, the gaps in the fissures by collagenase digestion appeared filled with a hyaline matrix of scarce cellularity, and the interface between cartilage and subchondral bone has become blurred, and the regular and continuous osteochondral junction was disappeared. In the normal control groups (F), TMJs had well-organized cartilage microstructures and trabecular connectivity, and the interface between cartilage and subchondral bone was assumed by a regular and continuous osteochondral junction.



**Figure 2.** AB-PAS staining in the cartilage and subchondral bone in the experimental group (A-E), the integrity of the osteochondral junction was broken by conjunctive runnings in the central part of the temporomandibular joint and by invaginations in subchondral bone region (A), the osteochondral junction had completely disappeared by active subchondral bone remodeling and the chondroid metaplasia was observed in the subchondral bone (C, D). The synthesis of chondrocytes was extremely active because acid mucopolysaccharides in chondrocytes and collagen of matrix components between trabecular bone were significantly enhanced compared with the control group (F).

electrophoresis, and then blotted. The designated proteins on the blots were assessed using primary antibodies against SOX-9, CD44 and  $\beta$ -actin, followed by horseradish peroxidase-conjugated IgG as the secondary antibody that was visualized using chemilumines-



Figure 3. The progenitor cells and bone marrow stem cells, adjacent to the damaged cartilage areas and in the subchondral bone regions, showed obviously increased immuno-staining for CD44 in the 2 weeks group.



Figure 4. Immunohistochemical staining of collagen type II showed that there were active collagen synthesis and metabolism in the area of positive staining in the 2, 4, 6 and 8 weeks groups.

cence agents. For each blot in 3 repeated experiments, protein band intensity was quantified using scanning densitometry.

#### Statistical analysis

Results were expressed as mean  $\pm$  standard error of mean (SEM). Differences between groups were analyzed using one-way ANOVA. In the case of two groups, Student's t-test was used. Statistical significance was assumed at a *P* value of < 0.01. Data were obtained from three independent experiments.

#### Results

#### Condylar cartilage morphology

*Control group:* In the control joints, the condylar surface was smooth and the cartilage showed

the typical organization in fibrous, proliferative, chondroblast, hypertrophic and erosive layers (**Figures 1** and **2**).

*Experimental group:* In most of the specimens, the gaps in the fissures by collagenase digestion appeared filled with a hyaline matrix of scarce cellularity, without proteoglycans or type II collagen. In all samples, the condylar cartilage adjacent to the edges of the fissures presented groups of a variable number of chondrocytes. Cartilage of the digested area displayed a reaction characterized by thickening of the cartilage cellular structure with retraction structure formation in the subchondral bone (**Figures 1** and **2**). This thickened cartilage maintained the shape of the condylar surface by filling the bone concavity that appeared in the central area.

# Chondrogenic capacity of progenitor cells in osteoarthritis



**Figure 5.** Immunohistochemical staining of SOX-9 showed that Chondrocytes, cartilage cells and related functional cells could be seen SOX-9 protein expression in the 2, 4, 6 and 8weeks groups, and especially in the area of cartilage metaplasia in subchondral bone in the 6 weeks group.



Figure 6. Immunohistochemical staining of MMP-9 showed that there were active matrix metabolisms in the subchondral bone remodeling region in the 2, 4 and 6 weeks group.

#### Subchondral bone morphology

*Control group:* In control group, condylar subchondral bone was composed of cancellous bone crossed by vascular channels. The interface between bone and cartilage was assumed by a regular and continuous osteochondral junction (**Figures 1** and **2**).

*Experimental group:* Most of the specimens focuses on chondroid metaplasia were observed in the subchondral bone, promoting its remodeling, which could develop to endochondral ossification and increasing subchondral bone size (**Figures 1** and **2**).

At 2 weeks, the integrity of the osteochondral junction was broken by conjunctive runnings in the central part of the temporomandibular joint

and by invaginations in subchondral bone region. Meanwhile, a lot of chondrocytes appeared in trabecular bones around the invaginations and trabecular bones become larger coarse and non-uniform arrangement.

At 4, 6 and 8 weeks, the osteochondral junction had completely disappeared by active subchondral bone remodeling. During this period, a large number of chondrocytes appeared in subchondral bone region. The synthesis of chondrocytes was extremely active because acid mucopolysaccharides in chondrocytes and collagen of matrix components between trabecular bones were significantly enhanced by ABPAS staining. With the prolonged healing time, the synthesis of acid mucopolysaccharides and collagen gradually reduced, which can be seen in 10 weeks group.



**Figure 7.** The columnar analysis chart of immunohistochemistry by the Image Pro Plus Soft revealed that CD44, collagen type II, SOX-9 and MMP9 differentially expressed in the experimental and control groups, and "Group significantly different (P < 0.01), and "Group obviously different (P < 0.05).



Figure 8. Whole cell lysates were subjected to Western blotting with the indicated antibodies.  $\beta$ -actin served as an internal control.

# Protein expression in the mandibular condylar subchondral bone

Immunohistochemistry analysis by the Image Pro Plus Soft revealed that collagen type II-positive cells, CD44-positive cells, SOX-9positive cells and MMP9-positive cells displayed brown staining in the periphery and cytoplasm. At 2 weeks, CD44 expressions in subchondral bone were most significantly increased in TMJOA compared with the normal control group and other groups (P < 0.01) (Figure 3). At 4, 6 and 8 weeks, the osteochondral junction had completely disappeared by active subchondral bone remodeling, and collagen type II, CD44, SOX-9 and MMP-9 expressions in active subchondral bone region were significantly increased in TMJOA compared with the normal control group (Figures 3-6). The measurements of Integral Optical Density (IOD) and the columnar analysis chart of immunohistochemistry by the Image Pro Plus Soft revealed that CD44, collagen type II, SOX-9 and MMP9 were expressed differentially in the experimental and control groups (Figure 7). In all experimental group, SOX-9 expression was manifest in chondrocytes and subchondral bone in

TMJOA (Figure 5). Western blot analysis revealed that CD44 expression significantly emerged in subchondral bone region at 2 weeks group (P < 0.01) (Figures 8, 9). Meanwhile, SOX-9 expression emerged in all group, and the intensity was increased in the experimental groups compared with the normal control group (P < 0.05) (Figure 9).

# Discussion

Our results showed that there were the articular cartilage damage and the subchondral bone repair reactions in the collagenase-induced TMJOA rabbit models. Topical destruction of cartilage by collagenase was slit-like, even through full-thickness cartilage. Meanwhile, with the destruction of aggravation and the repair response, boundary between cartilage and subchondral bone had not obvious, or disappeared. Furthemore, the integrity of the osteochondral junction was broken by conjunctive runnings in the central part of the temporomandibular joint and by invaginations in subchondral bone region. Notably, chondroid metaplasia in the subchondral bone was promoting its remodeling, and which could develop to endochondral ossification in the collagenase-induced TMJOA. Those can be explained by nonuniform chondrocytes with distinct volume distributing in the subchondral bone trabecular. Secondly, in the subchondral bone area, there was a significant increase in the number of trabecular bone, the volume of bone marrow cavity, and the number of vessels. Therefore, cartilage defects that extend to the subchondral bone exhibit some ability to repair via the formation of neocartilage, probably due to the release of bone marrow derived stem cells from the underlying subchondral bone. Insight into the inflammation, degeneration and bone remodeling in subchondral bone is anticipated to improve our understanding of the pathogenesis of TMJOA and help design clinically relevant strategies for tissue engineering.

We also found that the progenitor cells mainly lied in subchondral bone region close to articular cartilage, and they are activated early during TMJOA initiation and progression. In our experiments, CD44 protein expression revealed that the biological functions of progenitor cells and bone marrow stem cells were activated early in the disease progression. We have known that CD44 protein as cell surface receptor is the typical surface marker of multipotent mesen-



**Figure 9.** The columnar analysis chart of Western blotting by image Analysis, according to the different concentrations revealed that CD44 and SOX-9 differentially expressed in the experimental and control groups, and "Group significantly different (P < 0.01), and "Group obviously different (P < 0.05).  $\beta$ -actin served as an internal control.

chymal cell, and involved in more complex interactions that include cell motility and migration, differentiation, signal transduction, and gene transcription. Meanwhile, renovation and remodeling of subchondral bone were determined by MMP-9 protein expression. In 2, 4 weeks groups, CD44 protein and MMP-9 protein expressions in subchondral bone, especially at the blurred junction of cartilage and subchondral bone, were significantly increased in active subchondral bone region close to articular cartilage compared with the other groups. It can be proved that beneficial activation of progenitor cells and bone marrow stem cells in subchondral bone at early stage of TMJOA played an important role on renovation and remodeling of subchondral bone, and which was instructive in the choice of treatment time. The manifestations of the cells and factors in subchondral bone were the most active in the 2 weeks group, and the performance continued to be active until the 8th week. These results might give some hints on repairing bone and cartilage defects timing. However, a complex relationship between subchondral bone and articular cartilage during OA initiation and progression elicits an unresolved question with valuable therapeutical implications [12], so further researches are needed.

Chondrogenesis is a complex process, in which progenitor cells and growth factors work in concert to induce chondrogenic differentiation [13,

14]. SOX-9 plays a key role in chondrogenesis and controls the expression of aggrecan and type II collagen [15]. Several cytokines and transcription factors, including transforming growth factor (TGF-β), bone morphogenetic proteins (BM-Ps), fibroblast growth factors (FGFs), parathyroid hormonerelated peptide (PTHrP), and SOX proteins, contribute to the chondrogenic process [16]. TGF- $\beta$  and BMPs have been involved in chondrogenesis mediated by Smad transcription factors, which bind to TGF-ß receptors and are phosphorylated following binding to type II receptors [17]. Both BMP receptor-associated Smads (1, 5, and 8) and the TGF-β receptor-associated Smads (2 and 3)

are released into the cytoplasm upon phosphorylation and form a complex with Smad-4. which then translocates into the nucleus, where it regulates the expression of genes such as SOX-9 [18]. SOX-9 regulates the expression of a major cartilage matrix protein, type II collagen  $\alpha$ 1, and enhances aggrecan gene activity in chondrocytes. These observations indicate that SOX-9 is an important pathway for the chondrogenic differentiation process [19-22]. Our results also showed that SOX-9 played the key roles in chondrogenesis throughout. Stem cell-associated proteins analysis of CD44 and SOX-9 expressions by western blot in subchondral bone region revealed that the chondrogenic differentiation process was regulated due to progenitor cells and bone marrow stem cells.

To sum up, our results suggest that the beneficial activation of progenitor cells and bone marrow stem cells in subchondral bone at early stage of TMJOA played an important role on renovation and remodeling of subchondral bone. In addition, Cytokines of SOX-9 and CD44 play a key role in promoting the differentiation of progenitor cells.

#### Acknowledgements

This study was supported by grants from the Committee of National Nature Science Foundation (No. 81400532) in China, Jilin University's Outstanding Young Teacher Training Program (No. 419080500367), and the Program for Fundamental Research of Jilin University (No. 450060491134).

#### Disclosure of conflict of interest

None.

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