Expression and Significance of High-Mobility Group Protein B1 (HMGB1) and the Receptor for Advanced Glycation End-Product (RAGE) in Knee Osteoarthritis

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Background: This study was performed with the aim to explore the expression of high-mobility group protein B1 (HMGB1) and the receptor for advanced glycation end-product (RAGE) in knee osteoarthritis (KOA) and its clinical significance.

Material/Methods: A total of 108 synovial tissues selected from KOA patients were included in the experimental group. Seventy-five synovial tissues of knee joints, selected from patients who were clinically and pathologically confirmed without joint lesion, were included in the control group. The mRNA and protein expressions of HMGB1 and RAGE were determined by using RT-PCR and immunohistochemistry, respectively. Western blotting was used for measuring relative protein expression. An ROC curve was drawn to evaluate the diagnostic value of HMGB1 and RAGE for KOA.

Results: The positive cell number and positive expression intensity of HMGB1 and RAGE in synovial tissue was higher in the experimental group than in the control group. PI for HMGB1 and RAGE expression in KOA patients was positively correlated with clinical classification of X-ray films (P<0.05). HMGB1 and RAGE mRNA expressions, as well as relative protein expression of HMGB1 and RAGE in synovial tissue, were higher in the experimental group than in the control group (all P<0.05). The sensitivity of HMGB1 protein, RAGE protein, HMGB1 mRNA, and RAGE mRNA were 76.9%, 64.8%, 86.1%, and 64.8%, respectively; and the specificity was 100%, 96%, 74.7%, and 80%, respectively.

Conclusions: The protein and mRNA expressions of HMGB1 and RAGE are both increased in KOA patients, suggesting that they are involved in KOA.

MeSH Keywords: Gene Expression • HMGB1 Protein • Osteoarthritis, Knee

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Background

Osteoarthritis (OA), a progressively degenerative joint disease, is characterized by joint pain, stiffness, tenderness, effusion, crepitus, and variable degrees of inflammation without systemic effects [1]. Knee osteoarthritis (KOA) is a degenerative and chronic disorder of the knee joint caused by damage to hyaline cartilage. It is the most common type of arthritis, as well as the most common musculoskeletal disease, in individuals older than 65 years [2]. KOA involves progressive degeneration of articular cartilage and synovial membrane, but its etiology is not fully understood. During the past decade, the role of inflammatory mediators during the onset and progression of KOA and other types of osteoarthritis has been of great interest to researchers [3,4].

High-mobility group box 1 (HMGB1) is a nuclear DNA-binding protein, and can be passively released by necrotic cells, macrophages, or other myeloid cells in response to inflammatory stimuli [5]. HMGB1 can induce the production of cytokines and blood vessels, and plays an important role in proliferation, differentiation, and migration of cells [5]. HMGB1 may be extracellularly translocated and acts as an inflammatory mediator in tissue invasion and tissue repair [6]. The extracellular action of HMGB1 is mediated by several receptors, including the receptor for advanced glycosylation end-products (RAGE) [7]. Binding to HMGB1, RAGE is a transmembrane protein with 1 short cytoplasmic domain and 3 IgG-like extracellular domains [8]. Activation of RAGE is involved in the inflammatory response and leads to the development of chronic inflammatory diseases [9]. As an important pro-inflammatory mediator, HMGB1, along with its receptor, RAGE, has been associated with the onset and progression of a wide range of disorders, such as liver cancer and systemic lupus erythematosus, and may be involved in the pathogenesis of arthritis [6,10,11]. Few studies have investigated the role of HMGB1 and its receptor, RAGE, in KOA. This study was performed to explore the expression of HMGB1 and RAGE in KOA and its clinical significance.

Material and Methods

Study subjects

A total of 108 synovial tissues selected from KOA patients hospitalized between May 2013 and December 2014 and undergoing arthroscopic debridement or total knee arthroplasty (TKA) in the Department of Rheumatology, Yuhuangding Hospital affiliated to Qingdao University were recruited into this study as the experimental group. There were 63 male patients and 45 female patients, with mean age of 55.75 ± 9.54 years, age range 43–75 (24 patients aged ≤45; 84 patients aged >45). Of all the patients, 52 received left knees treatment and 56 had their right knees treated. All patients received X-ray examination, and according to the classification of examination results, severity of KOA was graded as I, II, III, and IV on the Kellgren-Lawrence scale [12]. Examination results showed that 9 patients were grade I, 39 were grade II, 48 were grade III, and 12 were grade IV. Clinical diagnosis of KOA was conducted in conformity with American College of Rheumatology guidelines [13]. Inclusion criteria were: (1) conformity with clinical diagnosis guidelines, severity above moderate and severe; and (2) no paracentesis and injection into knee joint cavity during the past half year. Exclusion criteria were: (1) past history of rheumatoid arthritis, rheumatic arthritis, systemic lupus erythematosus, or other connective tissue disorders; (2) past history of liver and kidney malfunction or autoimmune diseases; (3) use of non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids within 1 week before treatment; and (4) past history of knee joint infections, joint tuberculosis, or knee surgeries. At the same time, 75 synovial tissues of knee joints, selected from patients without history of joint diseases or gross lesions from traumatic injuries, cruciate ligament tears, discoid cartilage or meniscus injuries, and also clinically and pathologically confirmed without joint lesion, were enrolled as a control group, including 39 males and 36 females, with a mean age of 55.24 ± 9.27 years, age range 40–72. No statistical significance was seen in sex and age between the 2 groups (both P > 0.05). The study was approved by the Ethics Committee of Department of Rheumatology, Yuhuangding Hospital affiliated to Qingdao University. Written informed consent was obtained from all participants or their families.

Immunohistochemical staining and scoring

Tissue sections were put into an oven at 65°C for 30–50 min until the paraffin wax trickled down, and then were immersed in dimethylbenzene I and II for 10 min each, in 100% ethanol for 10 min each, in 95%, 90%, 80%, and 70% ethanol for 5 min each. In dimethylbenzene I and II for 10 min each, in 100% ethanol for 5 min each. The sections were rinsed 3 times for 3 min each in PBS. After removal of PBS, the sections were bathed in 0.1 M citrate buffer (pH 6.0) and autoclaved at high temperature and high pressure for 90 s to achieve antigen retrieval. After passive cooling, the sections were rinsed 3 times for 3 min each in PBS. After removal of PBS, the sections were bathed in 0.3% hydrogen peroxide in methanol for 10 min at room temperature to block endogenous peroxidase activity. The sections were rinsed 3 times for 3 min each in PBS. After removal of PBS, 60 μL primary antibodies (mouse monoclonal antibodies against HMGB1 and RAGE developed by Beijing Biosynthesis Biotechnology Co., Ltd.) were added to each section, which were then stored at 4°C overnight. The sections were taken out and rinsed 3 times for 5 min each in PBS. After removal of PBS, 60 μL HRP-labelled Goat Anti-Mouse/Rabbit IgG (Fuzhou Maixin Biotech Co., Ltd.) was added to each section, followed by incubation at 37°C for 30 min. After rinsing 3
### Table 1. Primer sequences of HMGB-1, RAGE and β-actin.

| Primer | Primer sequence | Length (bp) |
|--------|----------------|-------------|
| HMGB-1 | Upstream primer: 5'-AATACGAAAAGGATATTGGCT-3' | 226 |
|        | Downstream primer: 5'-GGCTAGAACAACCTAT-3' | |
| RAGE   | Upstream primer: 5'-AGGAGGAAGGAGGAGCGT-3' | 194 |
|        | Downstream primer: 5'-TGGCAAGTGGGGTTATACAG -3 | |
| β-actin| Upstream primer: 5'-CCTGGGCATGGAGCTCCTGT-3' | 289 |
|        | Downstream primer: 5'-AGGGCCGGACTCGTACAC-3' | |

HMGB1 – high mobility group box B1; RAGE – receptor for advanced glycated end-products.

### Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA of the above-mentioned tissue specimens were extracted in accordance with the instructions of TRIzol (Invitrogen). The concentration of RNA was evaluated with an ultra-violet spectrophotometer: $A_{260}/A_{280}=1.8~2.0$, concentration adjusted to 500 ng/μL. RNA AMV kit ver. 3.0 reagent kit (TaKaRa) was used to reverse transcribe RNA into cDNA in a 10-μL reaction system, and cDNA was stored at –20°C. Length of primer sequences amplified fragments can be found in Table 1. The total reaction system (20 μL), including 1 μL cDNA, 10 μL PCR buffer, 0.6 μL upstream and downstream primers each, and 7.8 μL DEPC-H2O, was added to the 1 μL cDNA. The mixture was treated in cycles: 94°C for 10 s, 94°C for 30 s, HMGB-1 (PTG) 58°C for 30 s, RAGE 60°C for 30 s, and 72°C for 45 s. After 40 cycles, the mixture was treated with RAGE at 72°C for 10 min. The Applied Biosystems 7500 system was used for PCR, the differences in expression of HMGB1 and RAGE mRNA in normal and KOA synovial tissue were observed, and this procedure was repeated 3 times. The differences were counted with 2$-^{\Delta\Delta C_T}$ method. The products from PCR were electrophoresed in 2% agarose gel under a constant voltage of 130 V for 30 min and imaged with the Bio-Rad agarose gel imaging system.

### Western blotting

Fresh specimens (100 mg) were pulverized under liquid nitrogen, and 100 μL lysis buffer was added. The specimens were then kept on ice for 30 min for full pulverization, and centrifuged for 15 min at 12 000 rpm at 4°C. The supernatant was pipetted and tested with nuclei acid/protein detector for the concentration of protein. A total of 300 μg protein was taken, a loading buffer containing β-mercaptoethanol (BME) was added, and the mixture was kept in a water bath for 5 min at 100°C to denature protein. A 10% resolving gel and a 4% stacking gel were formulated. Electrophoresis buffer was added into an electrophoresis tank, 10 μL extracted protein was put into every blot, and protein standard marker was added to the last lane of the tank. After electrophoresis, protein was loaded to polyvinylidene fluoride (PVDF) membranes in a semi-dry system, and 5% non-fat milk was added to block antigen for 1 h. The protein was put into TBST containing mouse monoclonal antibodies against HMGB1 (1:800 dilution) and against RAGE (1:2000 dilution) (developed by Abcam) to incubate for 2 h at room temperature, followed by membrane washing and detection of hybrid signals with ECL reagent. After the blots were pressed to the films, the X-ray films were exposed to light and the gel images were analyzed for integrated optical density quantification, where expression levels of HMGB1/ RAGE proteins were corrected by that of CAPDH protein. Assessment of results was
performed as follows: with gray value of bands being analyzed with Quantity One 4.62, relative expression of HMGB1/RAGE proteins = gray value of HMGB1/RAGE band/gray value of CAPDH in the same specimen, and the result is represented by $c\pm s$.

**Statistical analysis**

Statistical analysis was conducted with SPSS 19.0 Statistics software (SPSS Inc, Chicago, IL, USA). Measurement data are expressed as mean ± standard deviation ($\mu \pm s$). The $t$ test was used to compare differences between the 2 groups. Comparisons among multiple groups were performed using 1-way analysis of variance (ANOVA). Count data are represented in percentage or ratio and comparison was performed with the chi square test. A receiver operating characteristic (ROC) curve was drawn to evaluate the diagnostic value of HMGB1 and RAGE for KOA. All tests were 2-sided, with $P<0.05$ being considered statistically significant.

**Results**

**HMGB1 and RAGE proteins expression in synovial tissue by immunohistochemistry**

Immunohistochemical results showed that HMGB1 protein positive signal was mainly found in cytoplasm, with a small number of stained nuclei, as indicated by brownish yellow or chocolate brown granules. RAGE protein positive signal was mainly found in cytoplasm as indicated by brownish yellow or chocolate brown granules. In the synovial tissue of the patients in the control group, few HMGB1 proteins in synovial cells presented a positive signal, and fewer RAGE proteins presented a weakly positive signal. In the experimental group, expression of HMGB1 protein was mainly located in synovial lining cells, inflammatory cells from synovial tissue, and vascular endothelial cells, and expression of RAGE protein was mainly located in synovial cells and partly in histiocytes and inflammatory cells, with a significant rise in the number of positive cells and
intensity of positive expression in both HMGB1 and RAGE compared with normal synovial tissue. According to positive index (PI) results based on the intensity of positive expression, PI for HMGB1 and RAGE expression in KOA patients was positively correlated with clinical classification of X-ray films \((P<0.05)\), but the differences of HMGB1 and RAGE expression in KOA patients were not statistically significant by sex, affected side and age \((P>0.05)\) (Figure 1, Table 2).

### Expressions of HMGB1 and RAGE mRNA in experimental group and control group

Expressions of both HMGB1 mRNA and RAGE mRNA in synovial tissue were detected in both the experimental group and control group, with details of mRNA expression as shown in electrophoresis pictures. The synthetic cDNA was amplified through PCR with GAPDH primer and electrophoresed in 2% agarose gel. Bright bands could be observed under an ultra-violet lamp, which proved cDNA to be of good quality (Figure 2). The experimental group showed higher expression levels of HMGB1 and RAGE mRNA in synovial tissue than in the control group, with differences being statistically significant \((P<0.05)\) (Figure 3).

### Expression levels of HMGB1/RAGE proteins in synovial tissue detected by Western blotting

Expression of HMGB1 and RAGE proteins was detected in all synovial tissue samples, with expression levels significantly lower in the control group than in the experimental group, and the differences were statistically significant \((HMGB1: 0.331\pm0.105 \text{ vs. } 0.798\pm0.294, P<0.05; \text{RAGE: } 0.309\pm0.114 \text{ vs. } 0.612\pm0.268, P<0.05)\) (Figure 4).

### Diagnostic value of HMGB1 and RAGE in KOA

The sensitivity and specificity of HMGB1 protein for the diagnosis of KOA was 76.9% and 100%, respectively, and the area under the ROC curve was 0.924 (95%CI=0.889~0.960). The sensitivity and specificity of RAGE protein for the diagnosis of KOA was 64.8% and 96.0%, respectively, and the area under the ROC curve was 0.829 (95%CI=0.771~0.887). The sensitivity and specificity of HMGB1 mRNA for the diagnosis of KOA was 86.1% and 80%, respectively, and the area under the ROC curve was 0.802 (95%CI=0.741~0.864) (Figure 5).

### Discussion

Research in articular cartilage diseases has included a focus on changes in cartilage matrix, inflammatory mediators, and loss of cartilage chondrocytes during progression of OA. Among these research topics, the role of inflammatory mediators in KOA and other types of osteoarthritis have attracted...
increasing attention [3,4]. HMGB1, as a typical signal protein and pro-inflammatory mediator, plays a critical role in autoimmune response [6]. RAGE, easily binding to HMGB1, assisted the HMGB1 in the onset and progression of various chronic diseases [9]. Therefore, the objective of this study was to explore the expression of HMGB1 and RAGE in KOA patients and its clinical significance. According to the results of the present study, expression levels of HMGB1 and RAGE mRNA and proteins were relatively higher in KOA patients, which proves that both HMGB1 and RAGE are closely associated with the progression of KOA.

Results of this study presented a significant increase of positively stained HMGB1 and RAGE proteins in the synovial tissue in the experimental group compared to the control group. Western blotting results showed that the relative expression of HMGB1 and RAGE proteins in the synovial tissue of the control group was significantly lower than in the experimental group. As an early and late proinflammatory cytokine, extracellular HMGB1 has been demonstrated to play a pivotal role in the pathogenesis of some acute and chronic inflammatory diseases by binding to RAGE [14]. Previous studies suggested that HMGB1 can activate cells of the innate immune system,
causing the production of proinflammatory cytokines, chemokines, and adhesion molecules, thus leading to strong inflammatory response [15]. To serve as an inflammatory mediator, HMGB1 needs to bind to its receptor, RAGE, so that its signaling pathway is activated and HMGB1 and RAGE work in synergy [8]. The possible mechanism might be that HMGB1 combined with RAGE leads to activation of the NF-κB pathway, inducing transcription of multiple proinflammatory genes and upregulating leucocyte adhesion molecules, resulting in initiating and maintaining an inflammatory reaction cascade [16]. Some studies have suggested involvement of HMGB1 and RAGE in the pathological changes of patients with lupus nephritis and rheumatoid arthritis, with significantly higher expression levels of HMGB1 and RAGE in the experimental group [6,17]. In OA research, results showed that mRNA level was associated with the development and progression of OA [18]. A previous study has shown that higher levels of VEGF mRNA and protein are found in OA compared to controls [19]. The results of the present study demonstrated that HMGB1 and RAGE mRNA expressions in synovial tissue was higher in the experimental group than in the control group, which suggests that HMGB1 and RAGE mRNA expressions may be associated with the development of KOA.

Conclusions

This study provides evidence that the protein and mRNA expressions of HMGB1 and RAGE are both increased in KOA patients, suggesting that they are involved in KOA. However, several limitations of the present study should be taken into consideration. Firstly, the signal transduction of extracellular HMGB1 is

Figure 4. Expression levels of HMGB1 and RAGE proteins in the experimental and control groups ((A) HMGB1 and RAGE protein blot images; (B) HMGB1; (C) RAGE; * indicating comparison with the control group, P<0.05).

Figure 5. Diagnostic values of (A) HMGB1 and (B) RAGE in KOA.
closely related with RAGE, TLR2, TLR4, TLR9, and CD24-Siglec-10, but in this study we only investigated HMGB1 and its receptor, RAGE. Secondly, we did not explore the association of HMGB1 and the treatment of KOA, and this needs to be studied in future research. Thus, whether HMGB1 inhibitor can be used in the treatment of KOA needs verification by further studies.

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