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Variation patterns of two degradation enzyme systems in articular cartilage in different stages of osteoarthritis: Regulation by dehydroepiandrosterone

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ABSTRACT

Background: Osteoarthritis (OA) is a multifactorial degenerative joint disease in which the cartilaginous matrix of the articular joint is destroyed in a continuous process. We evaluated mRNA levels of cysteine proteinases/cystatin C system and urokinase plasminogen activator/plasminogen activator inhibitor-1 (uPA/PAI-1) system in articular cartilage and regulation by dehydroepiandrosterone (DHEA) in different stages of osteoarthritis (OA).
Methods: One hundred and eight rabbits underwent anterior cruciate ligament transection (ACLT) in the left knee, 54 received weekly intra-articular injections of DHEA (100 μmol/l) 0.3 ml 3 weeks after transaction as DHEA group. Thirty-six rabbits (18 from 2 groups respectively) were euthanized 6, 9, and 12 weeks after ACLT. All left knee joints were assessed by gross morphology and histology, meantime the gene expression from articular cartilage was analyzed.
Results: Cathepsins and uPA gene increased significantly 6 weeks and reached peak in the 9th week, while declined to extremely low levels 12 weeks after ACLT. Cystatin C decreased accompanied by OA progression, while PAI-1 expressed in the same trend with uPA. Additionally, these 2 enzyme systems were markedly suppressed by DHEA 6 and 9 weeks after ACLT but not in the 12th week.
Conclusion: The variation of these 2 enzyme systems was closely related to the progression of OA, and could be regulated by DHEA especially in the early and medium stages of OA.

1. Introduction

In the normal articular joint, cartilage homeostasis is maintained by a balance between the synthesis and degradation of articular cartilage composed of proteoglycans and type II collagen l [1,2]. However, in OA, the balance shifts toward catabolism, leading to cartilage destruction, which the cause of the disease is related to a complex interaction of mechanical and biochemical factors. Among various biochemical factors, the excessive production of proteolytic enzymes was regarded as the main factor. Those enzymes include metalloproteinases of the MMPs [3], ADAMTs families of proteases [4], cysteine proteases [5], and uPA/plasmin [6,7]. Traditionally, metalloproteinases have been considered as potential culprit enzymes over cysteine proteases and urokinase plasminogen activator. However, recent advances in the identification and characterization of novel cysteine proteases and urokinase plasminogen activator have attracted strong interest. Some new therapies focused on these two enzymes are also widely investigated to treat tissue degenerative and inflammatory processes [8–11].

Cysteine proteases are lysosomal enzymes of papain family, among which cathepsin K, B, L, and S are regarded as most relevant to the development of OA. The role of cathepsin K which degrades both type II collagen and aggrecan in articular cartilage in OA pathophysiology has been previously well documented [12–14]. High levels of extracellular cathepsin B found in the zones of hypercellularity and in chondrocytes suggest a leading role of the enzyme in the progression of OA [15,16]. Cathepsin L also has the collagenolytic activity and degrades basement membrane components [17], thereby facilitating cellular infiltration. And also cathepsin S is a potent cysteine protease that has the ability to degrade several extracellular matrix (ECM) proteins at neutral pH [18]. Cystatin C, which is a small protein (13.3 kDa), is known as the specific endogenous inhibitor of cysteine proteinase [8,19]. The imbalance of cysteine proteinases/cystatin C system could be an important contributing factor in development of OA [20]. For uPA/PAI-1 system, clinical and experimental evidence suggests that it contributes to joint pathogenesis in osteoarthritis and rheumatoid arthritis [21,22] and that the disturbed balance between uPA and PAI-1 ultimately contributes to the occurrence of arthritis disease and determines the extent of degradation of the extracellular matrix [23,24]. Moreover, enhanced levels of uPA/PAI-1 system have been associated with clinical severity of OA [6].

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Taken together, the important role of cysteine proteinases/cystatin C system and uPA/PAI-1 system during the development of OA has been recognized. However, there is little information about how these two systems change in different stages of OA. In addition, it is unknown whether dehydroepiandrosterone (DHEA), which plays a cartilage-protective role on experiment OA model in our and in others studies [25–27], can regulate these two systems during the development of OA. Therefore, the aim of the present study was to investigate the variation patterns of cysteine proteinases/cystatin C and uPA/PAI-1 system in different stages of OA and to determine the effects of DHEA on these two systems. The study induced OA using an anterior cruciate ligament (ACL) transection model in rabbits.

2. Methods

2.1. ACL transection and animal care

This study was approved by the Institutional Animal Care and Use Committee of Zhejiang University, Hangzhou, China. One hundred and eight New Zealand White rabbits (male, 2.0–2.5 kg, 3 month old) underwent ACL transection in the left knees. For the ACL transection, animals were first appropriately sedated and anesthetized. The left knee was then shaved, prepped, and draped in a sterile manner. A 3-cm medial parapatellar incision was made, and soft tissue was carefully dissected until the joint capsule could be opened. The patella was then dislocated, and the ACL was isolated and sharply divided. ACL transection was confirmed both visually and with Lachman testing by both the surgeon and an observer. The wounds were then closed and antiseptically treated. Rabbits were given appropriate postoperative care and analgesia. All animals were allowed normal cage activity.

Three weeks after ACLT, all animals were divided into two groups randomly, 54 rabbits in each group. One group without any treatment was severed as OA group, while the other group was intra-articularly injected with 100 μmol/l DHEA (Sigma, Fluka, St. Louis, MO) 0.3 ml in left knees 3 weeks after transection, once weekly. Thirty-six rabbits (18 from OA group and 18 from DHEA group) were killed 6, 9, and 12 weeks after ACLT respectively. The knee joints from two groups were assessed by gross morphology and histology. Articular cartilage from femoral condyles were analyzed for gene expression of cathepsin K, B, L, S, Cystatin C, uPA, and PAI-1 using real-time quantitative PCR. The right knees from untreated group were used as the control.

2.2. Macroscopic grading assessment of articular cartilage

After rabbits were killed, gross morphologic grading was performed immediately, using a India ink classification system, where the criteria used [28] were as follows: for grade 1 (intact surface), surface appeared normal and did not retain any ink; for grade 2 (minimal fibrillation), surface exhibited normal color before staining but retained the India ink as elongated specks or light gray patches; for grade 3 (overt fibrillation), surface was velvety in appearance and retained ink as intense black patches; and for grade 4 (erosion), loss of cartilage was evident, with exposure of the underlying bone. The femoral condyles were photographed using a high-resolution digital camera (IXUS960; Canon, Japan). After morphologic grading, the left knees (18 from OA group and 18 from DHEA group) were irrigated with saline solution to remove India ink and divided into 2 parts randomly. The first part (9 from OA group and 9 from DHEA group) was subject to histologic and histomorphometric evaluation, while the second part (left 9 from OA and DHEA groups respectively) was sampled and stored at −70 °C for RNA extraction and real-time quantitative PCR analysis.

2.3. Histologic assessment of articular cartilage

Histologic evaluation was performed on 3 sagittal sections of each femoral condyle as described previously [26]. The first section was obtained from the region of the most damaged articular cartilage, and the other 2 sections were obtained from regions that were equally distant from the first section point. After being fixed in 10% neutral buffered formalin for 48 h, the specimens were placed in 5% hydrochloric acid for 24 h. The decalcified tissue samples were then sequentially dehydrated in alcohol and embedded into paraffin blocks before being sectioned at 5 μm. The sections stained with Safranin O/fast green were then evaluated by 2 independent researchers who reevaluated the knee sections several times and were blinded to all groups. The final macroscopic scores of each femoral condyle were calculated by the formula followed OARSI assessment system [29] (magnification ×50; LMD6000; Leica, Germany), score = grade × stage, and in this formula, grade (scale 0–6) was defined as OA depth progression into cartilage (Fig. 1B) irrespective of its horizontal extent; stage (scale 0–4) was defined as the horizontal extent of cartilage involvement within one side of a joint compartment (Fig. 1A) irrespective of the underlying grade; score (scale 0–24) was defined as assessment of combined OA grade and OA stage. The final score of each specimen was based on the most severe histologic changes while the stage and grade might not come from a same section in each cartilage specimen.

2.4. Messenger RNA (mRNA) expression of cysteine proteinases/cystatin C and uPA/PAI-1 system by real-time quantitative PCR

The samples of cartilage were pulverized in liquid nitrogen, and total RNA was isolated using Tri reagent (Sigma, Aldrich) according to the manufacturer's instructions. After treatment for 20 min at 37 °C with 1 unit of DNase I (Sigma, Aldrich) to prevent genomic DNA contamination, 1 μg of total RNA was reverse transcribed using 10 pmol of random hexanucleotidic primers (Promega, Madison, WI), 0.5 mmol/l dNTPs, and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega) at 37 °C for 1 h. The reaction was stopped by incubation at 70 °C for 10 min. Then, the quantification of gene expression levels for cathepsin K, B, L and S, cystatin C, uPA, and PAI-1 were carried out by real-time quantitative PCR with the iCycler apparatus system (Bio-Rad, USA). iQ™ SYBR Green supermix PCR kit (Bio-Rad, USA) was used for real-time monitoring of amplification (5 ng of template cDNA, 45 cycles: 95 °C/15 s, 60 °C/15 s) with primers in Table 1. Using Qs18s (5′-GACGGACCAGAGCGAAAGC-3′) and Qa18s (5-CGCCAGTCGGCATCGTTTATG-3) primers, a parallel amplification of oyster 18s transcript (EMBL EU236696) was carried out to normalize the expression data of the targeted genes transcript. The relative level of targeted gene expressions is calculated for 100 copies of the 18s housekeeping gene following the formula: $n = 100 \times 2^{-(\triangle CT \text{ targeted gene} - \triangle CT \text{ 18s rRNA})}$.

2.5. Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analyses were performed with software, SPSS 12.0 for Windows. Evaluation of the data was based on one-way ANOVA. *P* values less than 0.05 were considered significant.

3. Results

3.1. Macroscopic grading in early, medium, and terminal stages of OA

Lesion analysis of femoral condyles in the period of 6, 9, and 12 weeks after ACLT showed increasing severity. Six weeks after ACL transection, the articular cartilage began to degrade relative to the normal control specimens, there were minimal velvet-like changes could be found in the condyle. The articular cartilage was further degraded in the 9th week

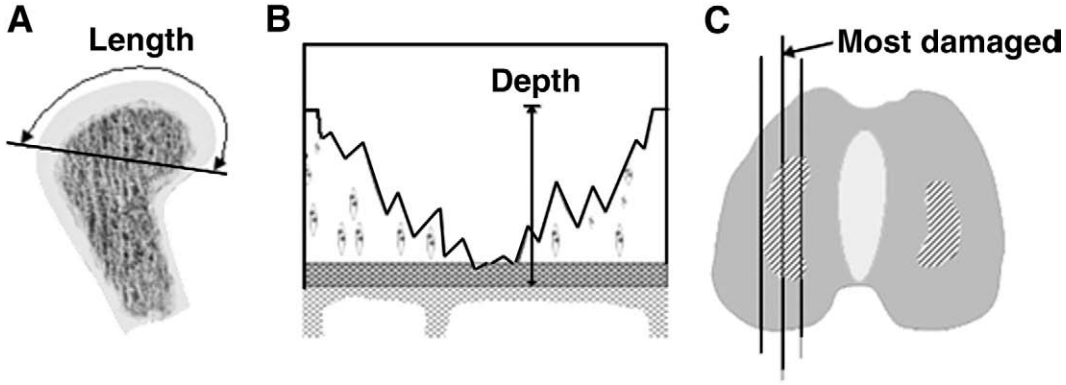

Fig. 1. Geometric parameters of the histologic specimen. Stage = extent of joint involvement, scale 0–4 (A); Grade = depth progression into cartilage, scale 0–6 (B); Score = stage × grade. At least 6 sections of each cartilage of femoral condyle were evaluated (C). The final score was based on the most severe histologic changes.