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ORIGINAL PAPER

Down-regulation of programmed cell death 5 by insulin-like growth factor 1 in osteoarthritis chondrocytes

Chengqing Yi · Chunhui Ma · Zongping Xie · Guoqiao Zhang · Wangsheng Song · Xiaokai Zhou · Yun Cao

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Abstract
Purpose The aim of this study was to investigate the expression of insulin-like growth factor (IGF)-1 and programmed cell death 5 (PDCD5) in osteoarthritis chondrocytes, and to explore the potential correlation between them in the apoptosis process of osteoarthritis chondrocytes.
Methods Patients with knee osteoarthritis were placed into four categories according to radiological staging. The mRNA and protein levels of IGF-1 and PDCD5 in osteoarthritis chondrocytes were respectively detected by quantitative reverse transcriptase polymerase chain reaction (qPCR) and western blotting. In addition, IGF-1 and PDCD5 protein expression in chondrocytes were also measured by immunohistochemistry. Apoptotic cells were measured by TUNEL staining.
Results Both the mRNA and protein levels of IGF-1 were down-regulated, while the levels of PDCD5 were up-regulated, and the mRNA and protein levels of IGF-1 were negatively correlated with those of PDCD5, respectively. The apoptotic cell was significantly increased in osteoarthritis chondrocytes compared with control. Importantly, the apoptosis rate was positively correlated with PDCD5 protein expression and negatively correlated with IGF-1 protein expression
Conclusions We concluded that IGF-1 may down-regulate the expression of PDCD5 and thus inhibit the apoptosis of osteoarthritis chondrocytes.

C. Yi (✉) · C. Ma · G. Zhang · W. Song · X. Zhou · Y. Cao
Department of Orthopaedics, Shanghai First People's Hospital,
No. 650 New Songjiang Road,
Shanghai 201620, China
e-mail: chengqingyiyiyi@hotmail.com

Z. Xie
Department of Orthopaedics, Shanghai Sixth People's Hospital,
Shanghai 200233, China

Introduction

Osteoarthritis (OA) is a chronic arthropathy characterized by degenerative lesions of articular cartilage and peri-articular bone proliferation. OA is the most common arthritis and is also one of the major causes of joint pain in elderly people [1]. Several factors have been implicated in the pathogenesis of OA. For example, epidemiological studies show that OA may be caused by the joint effects of genetic and environmental factors [2, 3]. Loeser suggests articular chondrocytes exhibit an age-related decline in proliferative and synthetic capacity while maintaining the ability to produce pro-inflammatory mediators and matrix degrading enzymes, which contribute to the propensity to develop OA [4]. Recent studies have shown the presence of apoptotic as well as anti-apoptotic mechanisms in OA cartilage [5]. The OA cartilage has a higher proportion of apoptotic chondrocytes than normal tissue [6, 7]. Increased chondrocyte apoptosis may be related to up-regulated expression of both Akt and PKCα in human OA cartilage [8]. ADAM15, a disintegrin metalloproteinase, is up-regulated in OA and further reduces downstream caspase 3/7 activity that controls cell fate and thus the integrity of the entire cartilage [9]. However, the apoptotic and anti-apoptotic mechanism of OA remains not well understood and further study is still needed.

Programmed cell death 5 (PDCD5), formerly designated as TF-1 cell apoptosis-related gene-19 (TFAR19), is first identified as a gene up-regulated in TF-1 cells that are undergoing apoptosis [10]. PDCD5 is located in chromosome 19q12-q13.1 and consists of six exons and five introns. PDCD5 has been demonstrated to be widely expressed within the organizations [11] and PDCD5 can promote the apoptosis process induced by a variety of compounds [12]. Increased expression and nuclear translocation of PDCD5 is also observed in rheumatoid arthritis patient-derived fibroblast-like synoviocytes undergoing apoptosis [13].

Insulin-like growth factor 1 (IGF-1) is a 70-amino-acid straight chain, basic peptide with homology to human pro-insulin [14]. It can be synthesized from bone, cartilage and a variety of the other tissues to promote mitosis onset [15]. IGF-1, up to 57.14 ng/mL, has the ability to promote the proliferation of chondrocytes in a dose-dependent manner [16]. Disruption of the IGF-1 gene in osteocytes impairs developmental bone growth [17].

In the present study, we aimed to investigate the expression of IGF-1 and PDCD5 in OA chondrocytes, and explore the potential correlation between them in the apoptosis process of OA chondrocytes.

Materials and methods

Materials

Dulbecco's modified Eagle's minimal essential medium was purchased from Life Technologies (USA). Newborn calf serum was obtained from GIBCO (Grand Island, NY, USA). Superscript II and total RNA extraction kit (Trizol) were purchased from Invitrogen (USA). Taqmanmaster mix was obtained from ABI (USA). Primers were synthesized by Invitrogen (USA). Anti-PDCD5 antibody was purchased from Abcam. Collagenase I and mouse monoclonal anti–β-actin antibodies were obtained from Sigma (USA). Liquid DAB was purchased from the Soledad Company (China). Electronic balance was obtained from Mettler Company. The high-speed low-temperature centrifuge was purchased from Beckman (USA). The inverted microscope was obtained from Olympus (Japan), and the Heraeus cell incubator was purchased from GB (USA). The 7500 fluorescent quantitative PCR instrument was obtained from ABI (USA).

The OA patients with total knee replacement surgery or synovectomy (n=39) and the normal fracture patients (n=15) were collected from March 2011 to January 2012 at the Joint Clinic Research Centre of Affiliated Hospital of Fudan University. All the patients with OA met the American Rheumatism Association revised diagnostic criteria [18].

Radiographic classification for knee osteoarthritis

For the documentation of knee damage based on radiological findings, four-grade classification was defined as follows: grade 1, doubtful narrowing of joint space and possible osteophytic lipping; grade 2, definite osteophytes and possible narrowing of joint space; grade 3, moderate multiple osteophytes, definite narrowing of joints space, some sclerosis and possible deformity of bone contour; grade 4, large osteophytes, marked narrowing of joint space, severe sclerosis and definite deformity of bone contour.

Isolation of chondrocytes

Cartilage fragments were cut into small pieces. After washing with PBS twice, cartilage pieces were digested with 5 ml 0.2 % type II collagen for six hours, at 37 ° C, in a 5 % CO_2 humidified incubator, then the cartilage suspension was harvested in sterile tubes containing 5 ml DMEM, and centrifuged at 1,500 rpm for five minutes. Finally, the cells were resuspended using DMEM. After straining with 200 mesh stainless steel screen, cells were seeded in T25 flasks at a density of 1×10^5 cells per flask.

Primary culture of isolated chondrocytes

After trypsinized, cells were seeded in T25 flasks at a density of $2–3\times10^5$/ml (ten flasks, each contains 2 ml cell suspension), then the cells were incubated at 37 ° C in a 5 % CO_2 humidified incubator. The cultures were examined for growth 72 hours later, and the culture medium was changed every two days.

Subculture of chondrocytes

The culture cells were examined using an inverted phase-contrast microscope. The culture media was removed and the cells were gently washed with PBS. Then, the cells were incubated with 2 ml 0.25 % trypsin at room temperature for five to ten minutes. Finally, the resuspended cells were seeded as described above, and the medium was refreshed every two days with subculturing.

Real-time quantitative PCR

Total mRNA of OA chondrocytes was extracted using TRIzol according to the manufacturer's instructions (Life Technologies, Rockville, MD, USA). A total of 1 μg RNA was converted to cDNA and stored at −80 °C. A total of 50 μL of reaction mixture was prepared for each sample, containing 25 μL Real-time PCR MasterMix, 2 μL primer mixture (10 μM each), 5 μL cDNA, and 16 μL RNase free water. The PCR amplification cycle was set as 50 °C for two minutes and 94 °C for two minutes for initial denaturation, and then performed for 40 cycles under the following conditions: 15 seconds at 95 °C, and one minute at 60 °C. Copy numbers were obtained through the extrapolation of Ct values of the test samples against the corresponding standard curve, and the housekeeping gene β-actin was used to normalize the amount of mRNA from each sample. The primer sequences are shown in Table 1.

Western blotting

Cells were washed with cold PBS three times and then lysed with 100 μL lysis buffer (20 mM HEPES, pH 7.4, 1 % NP-

Table 1 Primer sequences of analysed target genes

Primer	5′-3′
IGF-1-L	TGGTGGACGCTCTTCAGT
IGF-1-R	ACAATGCCCGTCTGTGGT
PDCD5-L	CGGAATTCACCATGGCGGACGAGGAGC
PDCD5-R	CGGAATTCAATAATCGTCATCTTCATC
β-actin-L	CTCCATCCTGGCCTCGCTGT
β-actin-R	GCTGTCACCTTCAeeGTTCC

40, 2 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM PMSF,1 mM TPCK, 2 mM Na_3VO_4, and aprotinin). About 30 μg total proteins were loaded in each well and separated by 15 % sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred onto polyvinylidene fluoride (PVDF) membrane. After electrophoretic transfer, membranes were incubated with the primary anti-bodies for one hour at 4 °C and the second antibody at 37 °C for one hour, and then the immunocomplexes were set up for ECL detection in dark room. β-actin was used for internal control.

TUNEL staining

The slides were rinsed with 0.1 mol/L HCL for 20 minutes, ethanol rinsing, and then dried naturally. Slides were incubated in a solution containing 3×SSC, 0.02 % Ficoll and 0.02 % polyvinylpyrrolidone at 65 °C for two hours. Subsequently, the slides were fixed with ethanol/acetic acid (3:1) for 20 minutes. After drying naturally, the slides were autoclaved. The treated slides were transferred into a petri dish and the chondrocytes were inoculated. The following culture conditions and time were the same as above. Apoptotic cells in each section were measured by TUNEL assay kit (Wako) according to the manufacturer's protocol. TUNEL-positive cells in three different areas were counted under a microscope (Axioskop 2 plus; Zeiss, Wetzlar, Germany). The cells with brown nucleus were recognized as positive. The apoptosis index (AI) was calculated as follows: AI=(positive cells/total cells)×100 %.

Immunohistochemistry

The slides were rewarmed at 37 °C for 45 minutes after incubating with 50 μl antibodies at 4 °C overnight. After washing with PBS three times (five minutes each time), the slides were incubated with 40–50 μL second anti-body at room temperature for one hour followed by rinsing in PBS three times (five minutes each time). The slides were observed under light microscope after incubating freshly prepared DAB solution for five to ten minutes. After rinsing with tap water for ten minutes, the slides were counterstained with hematoxylin for two minutes. Hydrochloric acid alcohol solution (1 %) was used for differentiation, and then the slides were rinsed with tap water for ten minutes. Finally, specimens were dehydrated and mounted, and examined with the microscope equipped with an image analysis system. The yellow or brownish-yellow staining represents positive staining.

Results

The mRNA expression levels of IGF-1 and PDCD5

Real-time quantitative PCR was used to detect the mRNA expression levels of IGF-1 and PDCD5 in chondrocytes of patients with different radiological staging. The results indicated that the mRNA level of IGF-1 was decreased, while the mRNA level of PDCD5 was gradually increased, accompanied by elevated radiological staging. Importantly, the mRNA level of IGF-1 was negatively correlated with the mRNA level of PDCD5 (R^2=0.9, P<0.05; Fig. 1).

Protein levels of IGF-1 and PDCD5

Western blotting was performed to determine the protein expression levels of IGF-1 and PDCD5 in chondrocytes of patients with different radiological staging. As expected, the levels of IGF-1 were also down-regulated, while the levels of PDCD5 were up-regulated in the chondrocytes isolated from our subjects with different radiological staging (Fig. 2).

Chondrocytes apoptosis

TUNEL positive cells were detected in both the control and osteoarthritis groups. Typical characteristics of apoptosis included the condensed chromatin and the chromatin gathered around the nuclear envelope. The apoptotic cells were significantly higher in the OA groups compared with the control group (Fig. 3).

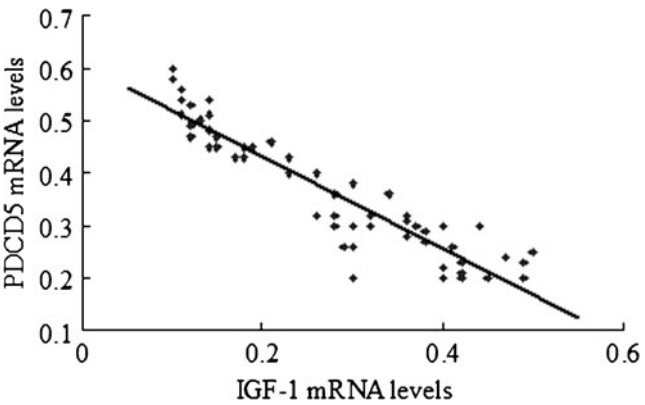

Fig. 1 Correlations between mRNA level of IGF-1 and PDCD5. The correlations between mRNA level of IGF-1 and PDCD5 were evaluated using the Spearman rank tests. The mRNA levels of IGF-1 were negatively correlated with the mRNA level of PDCD5

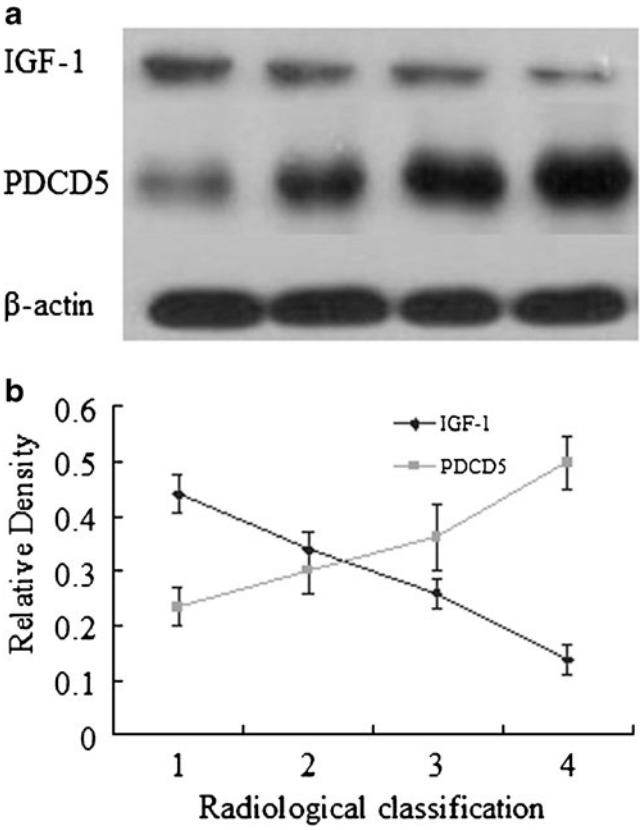

Fig. 2 Correlations between radiological staging and relative protein level of IGF-1 and PDCD5. **a** Protein levels of IGF-1 and PDCD5 were quantified by western blotting. Western blot analysis was performed using the corresponding antibodies to check expression of the proteins, and the β-actin was used as a loading control. The levels of IGF-1 were down-regulated, while the levels of PDCD5 were up-regulated in the chondrocytes isolated from our subjects with different radiological staging. **b** Correlations between radiological staging and relative level of IGF-1 and PDCD5 were evaluated using the Spearman rank test

The relationship between chondrocyte apoptosis and protein expression of IGF-1 and PDCD5

The cells with brown–yellow granules in the nuclei or cytoplasm were taken as PDCD5 positive cells. The PDCD5-positive cells were increased along the progression of OA grade (Fig. 4; Table 2). IGF-1-positive cells were decreased along the progression of OA grade (Table 2). The apoptosis rate of chondrocytes was negatively correlated with the levels of IGF-1 and was positively correlated with the levels of PDCD5 (Fig. 5).

Discussion

OA, also known as degenerative joint disease, is the most common type of arthritis. OA refers to a form of chronic joint inflammation caused by deterioration of joint cartilage, and poses a serious threat to human health [19, 20]. The pathogenesis of OA has not yet been fully elucidated until now. Previous study has indicated that multiple factors are involved in the pathogenesis of OA, the risk factors include increasing age, gender, race, trauma, obesity, genetics, immunity, inflammation, nutritional deficiencies, and lack of estrogen. Articular cartilage defects can speed up the process of joint degeneration, and eventually lead to arthritis. According to Homandberg et al., the occurrence of arthritis is probably due to the disruption of the collagen network caused by trauma. Cartilage cells are exposed to amounts of inflammatory factors, and the capability of the cartilage to resist external stress is weakened. Besides, cartilage matrix components are isolated from cartilage which can lead to more extensive degenerative changes [21]. The post-traumatic cartilage defect can induce the gradual degeneration of bone, cartilage, and surrounding tissues, which eventually lead to the formation of OA [22].

Apoptosis is considered a vital component of various processes including cell proliferation, proper development and normal cell turnover. The process of apoptosis can be divided into three stages: an initiation phase, a signaling phase and an execution phase. Apoptosis plays critical roles in development, maintenance of homeostasis, and host defense in multicellular organisms, and this process is controlled by multiple genes; therefore, apoptosis is also called programmed cell death (PCD) [23].

Cell death after traumatic cartilage may play an important role in developing OA and the repair of inflammatory joint

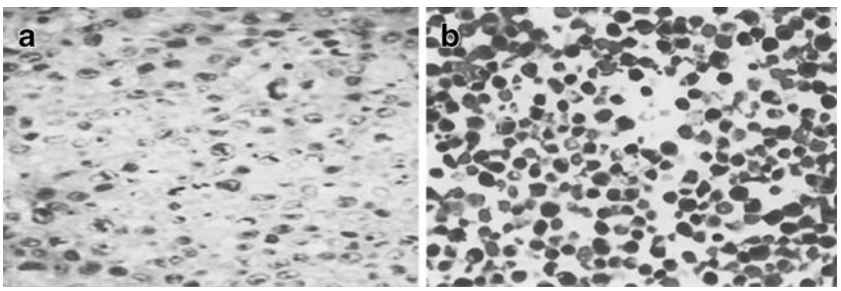

Fig. 3 TUNEL positive cells detected in both control and osteoarthritis groups. Chondrocytes were stained using a TUNEL assay kit. **a** Control group. **b** Osteoarthritis groups. Typical characteristics of apoptosis were condensed chromatin and chromatin gathered around the nuclear envelope (magnification, ×400)

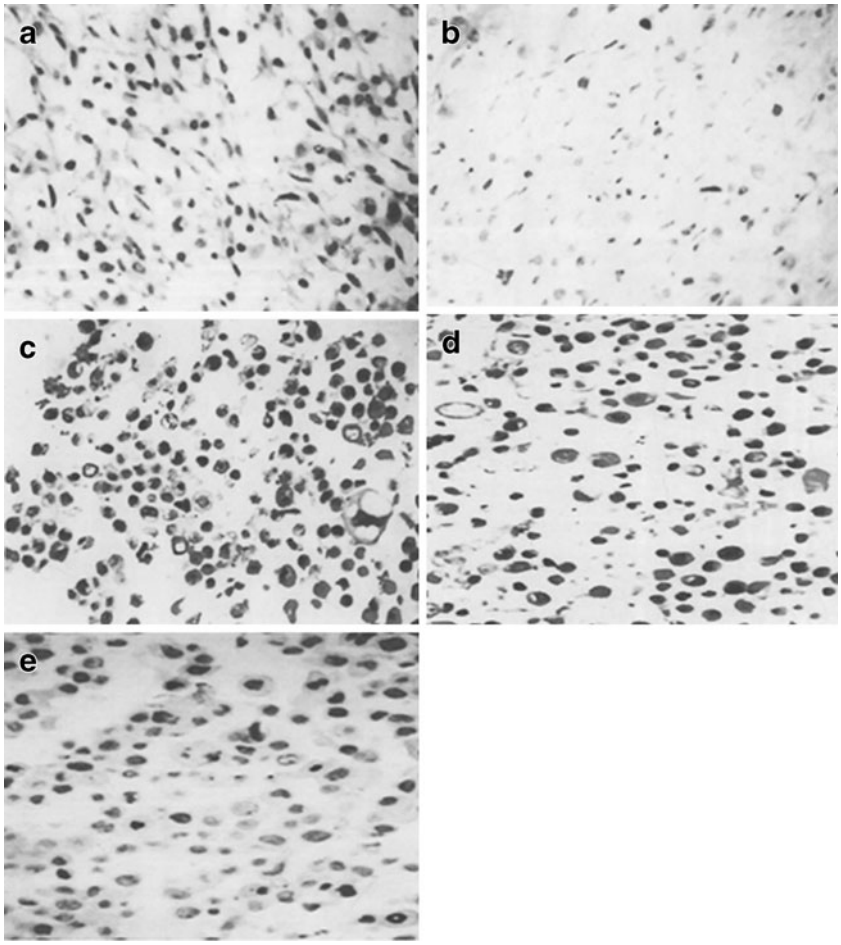

Fig. 4 Expression of PDCD5-immunoreactivity. Examples were examined under a light microscope equipped with an image analysis system. **a** Grade 1 according to radiological classification. **b** Grade 2 according to radiological classification. **c** Grade 3 according to radiological classification. **d** Grade 4 according to radiological classification. **e** The CK group. The cells with brown–yellow granules in the nuclei or cytoplasm were taken as PDCD5 positive cells (magnification, ×400)

disease. Two features of cartilage in skeletally mature individuals are important in considering the consequences of chondrocyte death. There are no macrophages in cartilage, meaning that the remnants of dead cells will persist in the cartilage matrix, which may potentially affect matrix structure and the function of viable chondrocytes. There are no vascular or mesenchymal stem cells in articular cartilage, so that precursor cells cannot be replenished. Chondrocytes are surrounded by a considerable extracellular matrix, and some chondrocytes also penetrate into the matrix, indicating that dead cells in the region can be hardly or only slowly regenerated, which can impair the ability of cartilage to maintain and repair extracellular matrix. Regeneration of damaged articular cartilage is very limited because cartilage lacks a blood supply, lymphatic drainage, and innervations. The cartilage defects will not be repaired when reaching a certain level of cell damage. The relationship between reduction of chondrocytes and increase of extensive degenerative changes indicates that cartilage cell death plays an important role in the pathogenesis of OA [24].

IGF-1 can stimulate proliferation of chondrocytes, promote the synthesis of collagen and proteoglycan, and play an important role in maintaining chondrocyte phenotype [25, 26]. IGF-1 may

Table 2 Expression of IGF-1 and PDCD5 in chondrocytes of patients with different radiological staging by immunohistochemistry

Expression	Grade 1	Grade 2	Grade 3	Grade 4
IGF-1	0.44±0.04	0.34±0.03	0.26±0.03	0.14±0.03
PDCD5	0.23±0.03	0.30±0.04	0.36±0.06	0.50±0.05

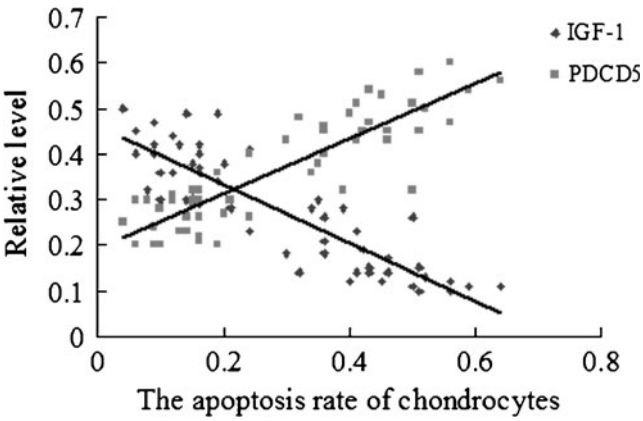

Fig. 5 Relationship between chondrocyte apoptosis and relative protein expression of IGF-1 and PDCD5. The correlations between the apoptosis rate of chondrocytes and relative protein level of IGF-1 and PDCD5 was evaluated using the Spearman rank test

promote the mRNA transcription, protein synthesis and the assembly of organelles, thus promoting cell proliferation [27]. According to Mor et al., IGF-1 is expressed in condylar cartilage, and appears to exhibit pleiotropic effects on osteoblasts [28]. IGF-1 stimulates metatarsal longitudinal growth reflected by the increased percentage of proliferating cells in the epiphyseal and proliferative zones, and IGF-1 is also involved in the differentiation and hypertrophy of chondrocytes [29]. Besides, IGF-1 modulates late G0/G1 progression by a posttranscriptional process that may involve protein modification [30].

PDCD5 is cloned as an increased expression gene whose expression is increased during the apoptotic process of TF-1 cells [31]. In the early stage of apoptosis, PDCD5 translocates from the cytoplasm to the nucleus, which is widespread during the apoptosis of cells, and does not depend on the cell types and apoptosis inducing factors [32]. Excessive proliferation and activation of synovial cells in patients with rheumatoid arthritis is due to an abnormal apoptosis process. Previous studies show that low rate of apoptosis is observed in cells of synovial tissue, the apoptosis rate of sublining stromal fibroblast-like cells is 3 %, and there are almost no apoptotic lining cells [33]. The lack of apoptosis cells can induce synovitis and synovial hyperplasia, leading to increased local pro-inflammatory cytokines and matrix metalloproteinases, eventually resulting in cartilage and bone destruction [34]. These abnormalities are related to abnormal expression of apoptosis-related genes, speculating that PDCD5 may play an important role in cells apoptosis.

In early OA, before collagen structure is destroyed, changes in chondrocytes and cartilage have been observed. The number of apoptotic cells correlates significantly with the severity of osteoarthritis. In addition, proteoglycan synthesis is significantly reduced, and cellular synthetic function is suppressed in chondrocyte apoptosis-existing regional. Therefore, the inhibition of chondrocytes apoptosis may be an effective strategy for osteoarthritis prevention and management [35].

In the present study, the expression of PDCD5 of osteoarthritis chondrocytes at both mRNA and protein levels was detected. The level of PDCD5 was negatively correlated with the rate of chondrocyte apoptosis, indicating PDCD5 may play a role in the regulation of chondrocytes apoptosis. However, molecular mechanisms of the role of PDCD5 in apoptosis of chondrocytes still remains unclear; further research is required to better understand the mutual relations of IGF-1 and PDCD5, and the role they play in apoptosis resistance or defectiveness.

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