Expression of PDCD5 , a novel apoptosis related protein, in human osteoarthritic cartilage[1]

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ABSTRACT

AIM: To investigate the expression features of PDCD5 (programmed cell death 5 protein) in osteoarthritic and normal human cartilage, and speculate on its potential functions in the pathogenesis of osteoarthritis (OA). **METHODS:** Articular cartilage specimens were obtained from 30 patients with OA and 16 healthy patients at the time of arthroplasty. Expression of PDCD5 was detected by flow cytometry, immunofluorescence, RT-PCR and immunohistochemical analysis. **RESULTS:** Enhanced expression and nuclear accumulation of PDCD5 in OA chondrocytes were found. PDCD5-positive chondrocytes were mainly distributed in the superficial and deep zones of OA tissue sections, as opposed to, in the superficial and middle regions of normal healthy tissue sections. **CONCLUSION:** Since apoptotic chondrocyte death occurs more frequently in OA cartilage than in normal healthy cartilage and PDCD5 is an apoptosis-related protein, the different expression patterns of PDCD5 in OA cartilage from that in normal healthy cartilage indicate that PDCD5 is involved in the pathogenesis of OA.

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease characterized by the progressive erosion of articular cartilage as well as the thickening of subchondral bone[1]. The pathogenesis of OA is poorly understood[2,3], however it is believed that both an imbalance between cartilage degradation and synthesis that leads to extracellular matrix damage as well as a severe loss of chondrocytes in the articular cartilage participate in the process. The loss of chondrocytes is at least partly due to apoptosis, a genetically controlled program of cellular self-destruction[4]. It has been reported that in OA cartilage 18 %-21 % of chondrocytes shows apoptotic features, compared with 2 %-5 % of normal healthy cartilage[5]. There are at least two independent pathways involved in promoting chondrocyte apoptosis in OA: one mediated by cytokines such as IL-1 and controlled by NO[6], another mediated by Fas ligand[7,8]. In addition, chondrocytes adjacent to cartilage defects expressed high levels of the apoptosis inhibitor Bcl-2[9]. These findings show that a highly complicated set of interactions are involved in the pathogenesis of OA.

We first cloned the TF-1 cell apoptosis related gene-19 (*TFAR19*) [designated Programmed Cell Death 5 (*PDCD5*) by the International Human Gene Nomination Committee] using a cDNA-representative differences analysis (cDNA-RDA) approach from TF-1 cells undergoing apoptosis[10,11]. Early studies have shown that

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PDCD5 can accelerate apoptosis of some tumor cells[12] and that PDCD5 expression is higher in apoptotic cells than that in normal cells. PDCD5 rapidly translocates from the cytoplasm to the nucleus of cells undergoing apoptosis, and nuclear accumulation of PDCD5 precedes the DNA fragmentation and phosphatidylserine (PS) externalization[13]. Introduction of an anti-PDCD5 antibody by *in situ* electroporation can suppress the apoptosis of Hela cells induced by etoposide, suggesting that the protein is critical for apoptosis[14]. Therefore, by comparing the characteristics of PDCD5 expression in OA cartilage with normal cartilage, we try to explore the possibility that PDCD5 participates in the apoptosis of OA chondrocytes.

MATERIALS AND METHODS

Clinical samples Nonosteophytic cartilage samples were obtained from 12 hip joints and 18 knee joints (mean age 64 years; range 45-72) with OA. Normal control samples without macroscopic and microscopic changes were taken from 6 knee joints and 10 patients with osteoporotic femoral neck fracture (mean age 66 years; range 56-78) undergoing joint replacement surgery. All patients had radiological features graded 4 or severe according to the Kellgren and Lawrence grading system for OA[15]. Prior to the study, we obtained informed consent from all patients and approval from the hospital ethics committee.

Isolation of chondrocytes Cartilage from each patient was carefully removed and pooled. The tissue was minced into small pieces (1-3 mm^3), washed in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA), and digested in 0.1 % collagenase II (Sigma, St Louis, MO) overnight at 37 ºC[16]. Cells were collected by centrifuging for 10 min at 1000×*g*, washed once with PBS, tested for viability (Trypan Blue exclusion test) and counted. Then the chondrocytes were fixed and kept at 4 ºC in PBS at a concentration of 4×10^6/mL[17].

Flow cytometry For scoring of apoptosis, freshly isolated chondrocytes (about 2×10^5/mL) were labeled with annexinV-FITC (20 mg/L) and propidium iodide (PI) (50 g/L) according to the manufacturer's instruction (ApoAlert AnenexinV-FITC Apoptosis Kit, Clontech). For detecting PDCD5 expression, mouse anti-human PDCD5 monoclonal antibody 3A3 (IgG) was prepared in our laboratory and labeled with FITC as described previously[13,18]. During labeling experiments, aliquots of 25 μL from a cell stock were permeabilized with 0.2 % Tween-20 (in PBS) for 15 min at 37 ºC. Subsequently, cells were blocked with PBS containing 2 % fetal calf serum (FCS) and incubated with FITC-3A3 IgG for 30 min at RT in the dark. Ten thousand cells were collected on a FACSCalibur flow cytometer equipped and analyzed using CellQuest software (Becton Dickinson).

Immunofluorescence Freshly isolated chondrocytes were seeded into 4-well chamber slides at a density of $3\times10^4/cm^2$ and incubated in DMEM for 24 h at 37 ºC in humidified air containing 5 % CO_2. The adherent chondrocytes were then fixed and permeabilized with 2 % paraformaldehyde and 0.1 % Triton X-100 in PBS for 30 min at RT. Cells were blocked and labeled as described in preparation for flow cytometric analysis for detecting PDCD5. Hoechst33258 (1 mg/L) was added for 30 min to allow visualization of the cell nuclei[19]. PDCD5 expression was analyzed by an Olympus fluorescence microscope and confocal laser scanning microscope (CLSM).

RNA extraction and RT-PCR RNA extraction from freshly isolated chondrocytes was done according to the Trizol protocol (Gibco BRL, Grand Island, NY, USA). Complementary DNA was prepared from 1 μg RNA using random hexamers and Superscript II RT (Gibco BRL). The resulting cDNA was stored at -80 ºC. Amplification of the PDCD5 cDNA was performed using recombinant *Taq* DNA polymerase with the following primers: 5'-CGG-AAT-TCA-CCA-TGC-CGG-ACG-AGG-AGC- 3' and 5'-CGG-AAT-TCA-ATA-ATC-GTC-ATC-TTC-ATC-3'. The housekeeping enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) was amplified as a positive control with the following primers: 5'-ACC-ACA-GTC-CAT-GCC-ATC-AC-3' and 5'-TCC-ACC-ACC-CTG- TTG-CTG-TA-3'. PCR products were electrophoresed on a 2 % agarose gel and visualized by ethidium bromide staining.

Immunohistochemical analysis Full thickness pieces of cartilage were dissected immediately after surgery. Samples were fixed with 10 % formaldehyde in PBS, decalcificated with 10 % formate, embedded in paraffin, and 5 μm sections were cut vertically. The tissue sections were placed on slides treated with 10 % polylysine, deparaffinized in xylene, rehydrated in graded series of ethanol, washed in PBS and the internal peroxidase was blocked with 0.3 % peroxide in methanol. Non-specific binding was blocked by incu-

bation of the sections with PBS containing 10 % normal goat serum for 30 min. Then tissue sections were incubated with the mouse anti-human PDCD5 monoclonal antibody 3A3 (IgG) (work concentration: 1.5×10^{-2} mg/L) overnight at 4 ºC. After washing twice in PBS slides were incubated with HRP conjugated goat anti-mouse IgG for 1 h at RT, subsequently, washed in PBS. Eventually, PDCD5-positive cells were displayed by reacting with diaminobenzidine (DAB) solution.

Sections from cartilage specimens were stained for PDCD5 in triplicates. The percentages of positively stained chondrocytes were determined by counting the positive and negative chondrocytes over entire area of one high power field (×400 magnification), containing between 80 and 120 cells.

Statistical analysis The SD in results are presented as means of multiple replicate cases within OA and control group and results are presented for a single representative experiment. Student's *t*-test was employed to assess the significance of difference between OA and control group. Statistical significance was set at $P<0.05$.

RESULTS

Flow cytometry Using flow cytometry, we examined the apoptosis features and PDCD5 expression of chondrocytes from OA and normal human articular cartilage. The apoptosis proportion of chondrocytes from OA cartilage specimens is different from that of normal cartilage, about 20 % and 5 %, respectively ($P<0.01$). Most of apoptotic cells were at the early stage of the process (Fig 1). We have also extracted the genomic DNA of both kinds of condrocytes and to research

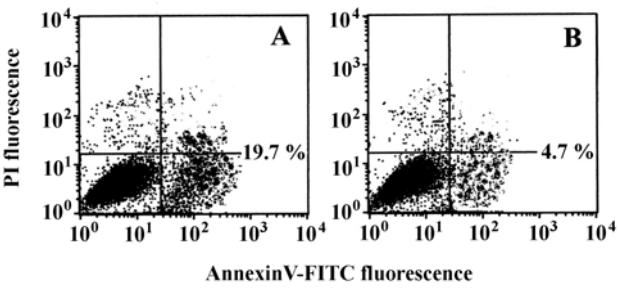

Fig 1. Detection apoptosis feature of chondrocytes by flow cytometry. Freshly isolated chondrocytes labeled with AnnexinV-FITC and PI were collected on flow cytometer and analyzed using the CellQuest software. Apoptotic chondrocyte death occurred more frequently in OA chondrocytes (A) compared with normal controls (B). This figure is a representation of 10 independent analysis. It is significantly different between OA (20.23 %±1.13 %) chondrocytes and control cells (4.85 %±0.19 %) ($P<0.01$).

the DNA fragmentation, but no typical DNA ladder in either group was found. Labeling chondrocytes with FITC-3A3 IgG, we found PDCD5 expression was significantly higher in chondrocytes from OA cartilage than normal control ($P<0.01$) (Fig 2).

RT-PCR To compare the transcription level of PDCD5 in OA and normal chondrocytes, RNA was extracted and RT-PCR was performed. The relative intensity of PCR products was detected with the Genetools software. An enhanced transcription of PDCD5 in OA cartilage compared with normal control was found (Fig 3), performed in three cases and two cases, respectively.

Immunofluorescence Direct immunofluorescence was used to detect PDCD5 expression level and location in chondrocytes. Almost all of the chondrocytes from both groups expressed PDCD5, while it was highly

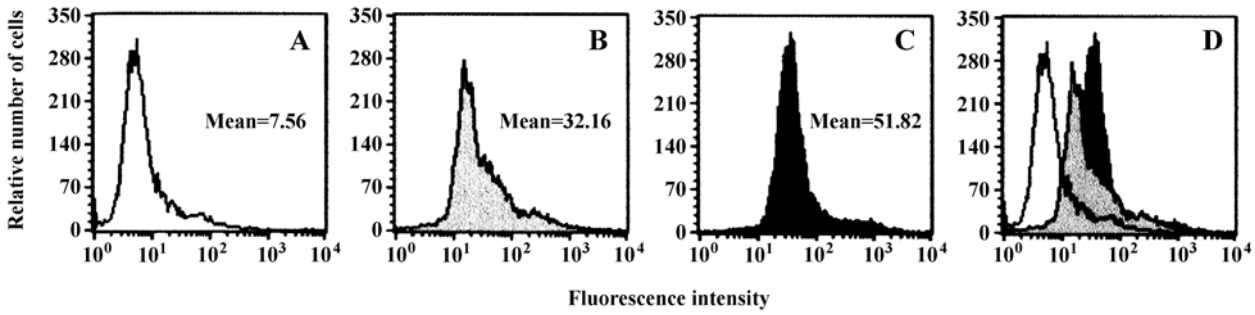

Fig 2. Flow cytometric analysis of the PDCD5 expression in chondrocytes. Fixed chondrocytes were permeabilized and stained with FITC-3A3 IgG and detected by flow cytometry. This figure is a representation of 10 independent analysis. It is significantly different between OA chondrocytes (51.82±2.04) and control cells (32.16±1.47) ($P<0.01$). A) normal chondrocytes without staining; B) normal chondrocytes stained with FITC-3A3 IgG; C) OA chondrocytes stained with FITC-3A3 IgG; D) overlay of A, B and C.

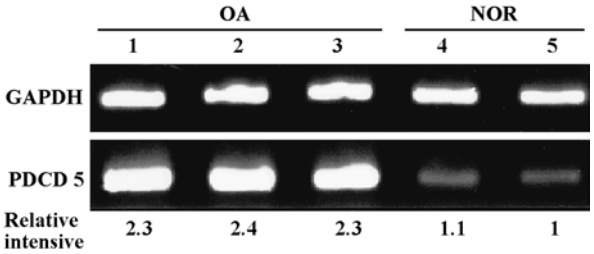

Fig 3. Detection of PDCD5 mRNA transcription in chondrocytes. RNA was extracted, RT-PCR for PDCD5 and GAPDH was performed. Lanes were scanned and intensity of the bands was determined using the Genetools software. Band intensities indicated an enhancement of transcription in OA patients. Representative samples of OA (three cases, Lane 1 to3) and normal (NOR) (two cases, 4 to 5) cartilages are shown.

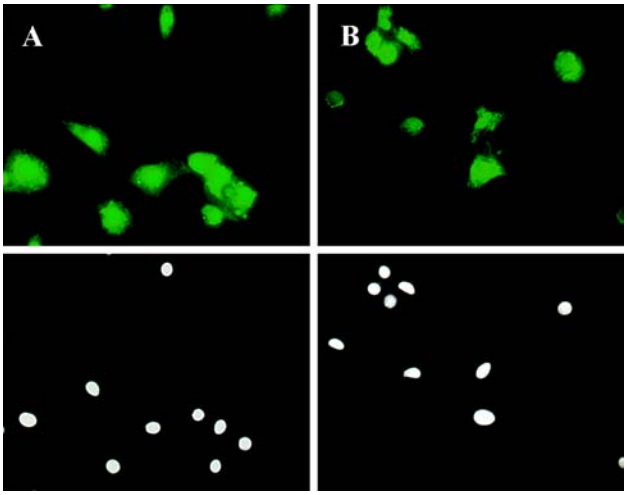

Fig 4. Detection of PDCD5 expression in chondrocytes by direct immunofluorescence. Chondrocytes were fixed, permeabilized, and labeled with FITC-3A3 IgG and counter stained with Hoechst33258 to visualize the nuclei. This figure is a representation of four independent analysis. PDCD5 is highly expressed in OA chondrocytes (A) compared with control cells (B). (Original magnification × 400).

expressed in OA chondrocytes (Fig 4). CLSM analysis showed that PDCD5 protein was intensively expressed and mainly localized in the nuclei of OA chondrocytes, in contrast, it was weakly expressed and showed perinuclear localization of normal controls (Fig 5). In the supernatant we found more chondrocytes which showed increased expression and nuclear accumulation of PDCD in OA group than in control group.

Immunohistochemical analysis Immunohistochemical method was used to detect the PDCD5 expression features in full-thickness cartilage. Using a monoclonal antibody specific to PDCD5, we demonstrated

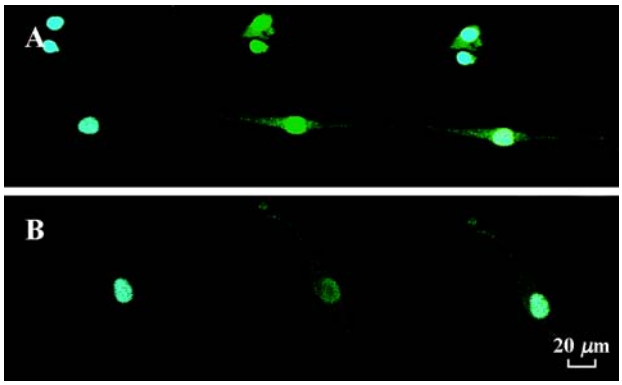

Fig 5. CLSM analysis of precise location of PDCD5 in the chondrocytes. Chondrocytes were fixed, permeabilized, and labeled with FITC-3A3 IgG (middle column) and counter stained with Hoechst33258 (left column) to visualize the nuclei. This figure is representation of four independent analysis. PDCD5 protein was intensively expressed and mainly localized in the nuclei of OA chondrocytes (A), in contrast, it is weakly expressed and showed perinuclear localization of control cells (B).

that there were more positive cells (51.75 %±3.92 %) in OA cartilage and their signals were more intensive than those (27.63 %±1.69 %) of normal control cartilage (n=14. Mean±SD. P<0.01). Additionally, PDCD5-positive cells mainly localized in the superficial and deep zone of OA cartilage, but in the superficial and middle

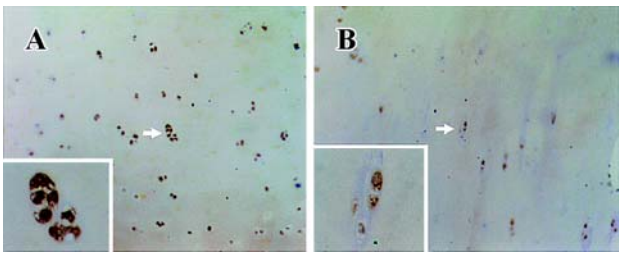

Fig 6. Immunohistochemical analysis of PDCD5 expression in cartilage sections. Samples were fixed, decalcificated, embedded in paraffin, and 5 μm sections were cut vertically covering full thickness of cartilage. The sections were stained with mouse anti-human PDCD5 monoclonal antibody 3A3. This figure is a representation of eight independent analysis. Positive cells were colored brown, using ×100 magnification for the overviews and ×400 magnification of positive cells (insets). There were more positive cells in OA cartilage (A) than in normal control cartilage (B).

layer of normal control cartilage (Fig 6).

DISCUSSION

Apoptotic chondrocytes have been detected in knee

articular cartilage obtained from patients of OA[20,21], and there is other evidence that chondrocyte loss may be important in the disease[22]. Since PDCD5 expression is higher in apoptotic cells than in normal cells and PDCD5 can accelerate apoptosis of some tumor cells[12], it is an interesting proposition that PDCD5 might be involved in the pathogenesis of OA.

Using different methods, we found higher levels of PDCD5 expression and apoptosis proportion in OA chondrocytes as compared with normal controls. Using immunofluorescence and CLSM analysis, we found that PDCD5 was more intensive in the nucleus of most OA chondrocytes. Since nuclear translocation of PDCD5 may be an early signal for apoptosis[13], these findings provide further evidence that PDCD5 may exert some functions on the OA apoptosis process.

We also found that most of the PDCD5-positive chondrocytes localized in the superficial and deep zones of OA cartilage, while localized in the superficial and middle layer of normal cartilage. According to the pathophysiology of OA, PDCD5 may accelerate apoptosis of chondrocyte, which results in superficial and deep zone cartilage lesions accompanied with a hypertrophic reaction in the subchondral bone of OA cartilage.

PDCD5 has been reported to be conservation in the process of evolution and a constitutive protein in many kinds of cells and upregulated during apoptosis process[10,11]. Using immunofluorescence, a sensitive method, we found PDCD5 was expressed in almost all of the chondrocytes with different intensities and location. Since less sensitive, immunohistochemical analysis can only display the cells which express PDCD5 much intensively. The higher proportion of positive chondrocytes from OA cartilage sections than that from normal sections implies the enhanced gene expression in disease.

Recombinant PDCD5 facilitates the opening of isolated mitochondrial pores, leading to a decrease in membrane potential and release of cytochrome C. This indicates that PDCD5 may be involved in a positive feedback loop that promotes mitochondrial apoptosis[23]. Since mitochondrial dysfunction can mediate several specific pathogenic pathways implicated in OA[24], it is deserved to study the role of PDCD5 on chondriosome of chondrocytes.

This is the first report comparing expression of PDCD5 in OA with normal cartilage *in vivo* and *in vitro*. The results showed an enhancement of PDCD5 expression and nuclear accumulation in chondrocytes from OA cartilage compared with normal control. Further study is necessary to elucidate the role of PDCD5 on chondrocytes and to determine whether it might be a new target for therapy of OA.

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