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Inflammation induction of Dickkopf-1 mediates chondrocyte apoptosis in osteoarthritic joint

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Summary

Objective: Dysregulated Wnt signaling appears to modulate chondrocyte fate and joint disorders. Dickkopf-1 (DKK1) regulates the pathogenesis of skeletal tissue by inhibiting Wnt actions. This study examined whether DKK1 expression is linked to chondrocyte fate in osteoarthritis (OA).

Method: Articular cartilage specimens harvested from nine patients with knee OA and from six controls with femoral neck fracture were assessed for DKK1, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), Bad, Bax, Bcl2 and caspase-3 expression by real time-polymerase chain reaction (RT-PCR) and immunohistochemistry. Apoptotic chondrocytes were detected by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labelling (TUNEL) and 4′, 6-dianidino-2-phenylindole dihydrochloride (DAPI) staining. Human chondrocyte cultures were treated with recombinant IL-1β and monoclonal DKK1 antibody to determine whether DKK1 impairs chondrocyte survival.

Results: Expression of DKK1 correlated with inflammatory cytokine levels (IL-1β and TNF-α expressions), proapoptosis regulators (Bad and caspase-3 expressions) and TUNEL staining in OA cartilage tissues. The IL-1β induced expressions of DKK1, Bax, Bad and caspase-3-dependent apoptosis of chondrocyte cultures. Neutralization of DKK1 by monoclonal DKK1 antibody significantly abrogated IL-1β-mediated caspase-3 cleavage and apoptosis and reversed chondrocyte proliferation. Recombinant DKK1 treatment impaired chondrocyte growth and promoted apoptosis. By suppressing nuclear β-catenin accumulation and Akt phosphorylation, DKK1 mediated IL-1β promotion of chondrocyte apoptosis.

Conclusion: Chondrocyte apoptosis correlates with joint OA. Expression of DKK1 contributes to cartilage deterioration and is a potent factor in OA pathogenesis. Attenuating DKK1 may reduce cartilage deterioration in OA.
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Key words: Osteoarthritis, Chondrocytes, Inflammation, Apoptosis, DKK1.

Introduction

Osteoarthritis (OA), a common cause of musculoskeletal disability in developed countries[1], is characterized by cartilage attrition, subchondral bone remodeling, osteophyte formation and synovial inflammation. Cartilage deterioration caused by inappropriate mechanical loading[2], disturbed biochemical regulation[3] and genetic mutation[4] is a potent etiologic causes of OA.

Impaired chondrocyte survival in joint microenvironments contributes to OA pathogenesis[5]. Perturbed homeostasis caused by inflammation[6], oxidative stress[7], mitochondrial dysfunction[8] and proapoptotic/antiapoptotic dysregulation[9] is known to modulate chondrocyte fate in the progression of OA. However, the molecular mechanisms underlying the programmed death of chondral cells are not well defined.

Wnt/β-catenin signaling components are essential for regulating cartilage development and the chondrocyte function[10–12]. Dysregulated Wnt proteins and destabilized β-catenin signaling are known to modulate the pathogenesis of human joint disorders[13]. Perturbation of Wnt inhibitor is reportedly a major contributor to osteoarthritic joint disorders. Osteoarthritic tissue is known to display intense gene expression and immunoreactivity of Wnt antagonists (secreted frizzled-related proteins; sFRPs)[14,15]. The sFRP3 gene mutations have also been linked to hip OA in women[16].

Secreted DKK1 suppresses the biological actions of Wnt proteins[17]. This Wnt inhibitor reportedly modulates the development and remodeling of various tissues. Dickkopf-1 (DKK1) is a potent osteogenesis-inhibiting factor that mediates bone loss induced by cancer cells, excess glucocorticoid and estrogen depletion[18–21]. Increased DKK1 signaling inhibits chondrogenesis in new bone formation[22]. Although Wnt antagonist signaling is known to accelerate cartilage deterioration, the biological role of DKK1 in chondrocyte fate in OA pathogenesis has not been elucidated. We hypothesized that DKK1 signaling may affect OA pathogenesis.

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This study investigated the spatial expression of DKK1 and inflammatory cytokines in the articular cartilage of patients with and without OA and examined whether DKK1 expression is linked to inflammatory cytokine induction of chondrocyte apoptosis.

Materials and methods

PATIENTS

This study was approved by the Institutional Review Board of this hospital, and informed consent was obtained from all subjects. The articular knee cartilage specimens were obtained from nine patients (all female; 70.9 ± 9.0 years old) with end-stage OA requiring total joint arthroplasty. Biopsy was performed on articular cartilage specimens from the hips of six femoral neck fracture patients who had undergone bipolar hip replacement (five females and one male; 68.8 ± 10.8 years old). Primary chondrocyte cultures were obtained from articular cartilage from three knee OA patients who had undergone arthroplasty (all female; 65, 67 and 75 years old) and from two female patients with femoral neck fracture (64 and 79 years old).

PRIMARY CHONDROCYTE CULTURES

Under aseptic conditions, cartilage specimens were sliced, minced, and incubated in 0.05% trypsin (Sigma−Aldrich Inc., St. Louis, MO) for 25 min at room temperature. The cartilage fragments were further incubated in cartilage dissecting medium (Ham's F12, 4 μg/ml G418, 10 μg/ml vancomycin and 30 μg/ml ceftazidim; Sigma−Aldrich Inc.) containing 0.3% collagenase (Sigma−Aldrich Inc.) for 30 min then incubated in cartilage dissecting medium containing 0.06% collagenase at 37°C for 16 h. After filtering the mixtures through a 70-μm filter, the cells were re-suspended in growth medium (Dulbecco's modified Eagle's medium (DMEM) and Ham's F12, 10% fetal bovine serum, 4 μg/ml G418) until subconfluent. The first two passages of cell cultures were used for subsequent experiments.

REAL TIME-POLYMERASE CHAIN REACTION (RT-PCR)

Tissues were ground with pestle and mortar in liquid nitrogen under RNase-free conditions. Total RNA from tissues and cell cultures was extracted with Tri reagent containing monophasic solution of guanidine thiocyanate and phenol (Sigma−Aldrich Inc.); 1 μg total RNA was reverse-transcribed at 50°°C for 2 min and then at 60°C for 30 min. Template (equivalent 20 ng total RNA) was amplified at 95°C for 5 min followed by 40 cycles of PCR reaction at 94°C for 20 s and 60°C for 1 min using 2X iQ™ SYBR Green Supermix and the iCycler iQ® Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The following gene-specific primers were used: DKK1 (forward, 5′-GAG TCC TTC TGA GAT GAT GG-3′; reverse, 5′-TTG ATA GCG TTG GAA TTG AG-3′; 141-base pair expected); interleukin-1β (IL-1β) (forward, 5′-ACG ATG CAC CTG TAC GAT CA-3′; reverse, 5′-TCT TTC AAC ACG CAG GAC AG-3′; 226-base pair expected); tumor necrosis factor-α (TNF-α) (forward, 5′-TCC TTC AGA CAC CCT CAA CC-3′; reverse, 5′-CAG GGA TCA AAG CTG TAG GC-3′; 208-base pair expected); Bad (forward, 5′-CCG AGT GAG CAG GAA GA CTC-3′; reverse, 5′-GGT AGG AGC TGT GGC GAC T-3′; 205-base pair expected); Bax (forward, 5′-GCT GGA CATTGG ACT TCC TC-3′; reverse, 5′-CAC TGT GAC CTG CTC CAG AA-3′; 181-base pair expected); caspase-3 (forward, 5′-TGG AAT TGA TGC GTG ATG TT-3′; reverse, 5′-GGC AGG CCT GAA TAA TGA AA-3′; 203-base pair expected); Bcl2 (forward, 5́-TTG TTC AAA CGG GAT TCA CA-3′; reverse, 5′-GAG CAA GTG CAG CCA CAA TA-3′; 176-base pair expected) and *18S* rRNA (forward, 5′-GTA ACC CGT TGA ACC CCA TT-3′; reverse, 5′-CCA TCC AAT CGG TAG TAG CG-3′; 153-base pair expected). The arbitrary intensity threshold (Ct) of amplification was also computed. Relative expression level was defined as $2^{(-\Delta Ct)}$, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{18S}$. In cell culture experiments, the fold change in mRNA expression was defined as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\text{treatment}} - \Delta Ct_{\text{vehicle}}$.

IMMUNOHISTOCHEMISTRY

Specimens were fixed in 4% PBS-buffered formaldehyde, decalcified in 10% phosphate-buffered saline (PBS-buffered) ethylenediaminetetraacetic acid (EDTA), embedded in paraffin, longitudinally cut into 5-μm sections and then transferred onto poly-lysine-coated slides. Immunoreacitivity was then tested by respective antibodies against DKK1, IL-1β, TNF-α (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and cleaved caspase-3 (Cell Signaling Technology Inc., Beverly, MA) using a non-biotin horseradish peroxidase detection system (BioGenex Laboratories Inc., San Ramon, CA) followed by counterstained with hematoxylin, dehydration and mounting. Those without primary antibodies were enrolled as negative controls for the immunostaining. Ten images were randomly selected from two sections of each specimen and the stained cells were counted under 400× magnification using a Zeiss Axioskop 2 plus microscope (Carl Zeiss Microimaging Inc., Gottigen, Germany) with a cool charge-coupled device camera and the Image-Pro® Plus image analysis software (SNAP-Pro *cf.* Digital kit; Media Cybernetics Inc., Silver Spring, MD). The positive immunolabeled and total cells per high power field in each section were counted, and the percentage of positive labeled cells was calculated.

CELL APOPTOSIS

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate-biotin nick end-labelling (TUNEL) was performed to detect apoptotic cells in the cartilage specimens by *In Situ* Cell Apoptosis Detection kits (Roche Diagnostics, East Sussex, UK) according to the manufacturer instructions. Briefly, deparaffinized sections were permeabilized using 0.1% Triton-X 100 for 20 min. Sections were probed with reaction buffer containing fluorescein-labeled deoxyuridine triphosphate (dUTP) and TdT for 60 min then incubated with fluorescein antibody conjugated with alkaline phosphatase for 30 min. After PBS rinsing, sections were subsequently developed with substrate solution containing fast red. Sections without primary TdT were designated as negative controls. Ten images were randomly selected from two sections of each specimen and the stained cells were counted under 400× magnification. In cell culture experiments, cultured chondrocytes were stained with 4′,6-dianidino-2-phenylindole dihydrochloride (DAPI; 2 μg/ml, Sigma−Aldrich Inc.) at 37°C for 30 min. Apoptotic cells exhibiting condensed or fragmented chromatin were recognized with dual TUNEL and DAPI staining. For each group in three repeated experiments, nine images were randomly selected under 200× magnification using an Axiovert 200 inverted fluorescence microscope. An Axiocam HRM cool charge-coupled device camera and an Axio Vision 4 image-analysis software (Carl Zeiss, Gottingen, Germany) were used for imaging. The apoptotic cells and total cells were counted, and the percentage of dual stained cells in each image was calculated.

CELL CULTURES

Primary human chondroyctes were cultured in DMEM/Ham's F12 (1:1, v/v) with 10% fetal bovine serum and 4 μg/ml G418. Human fetal chondrocytes (CRL-2847; American Type Culture Collection, Manassas, VA) were cultured in DMEM with 10% fetal bovine serum and 0.1 mg/ml G418 in a 5% CO_2, 37°C incubator until 80% confluent. Cells (1×10^5 cells/well, 6-well plate) were treated with recombinant human IL-1β (5, 10 and 15 ng/ml; R & D Systems Inc., Minneapolis, MN) and recombinant human DKK1 (100, 200 and 400 ng/ml; R & D Systems Inc.) for 12 h, 24 h, and 48 h. In some experiments, cell cultures were pretreated with 10 μM caspase-3 inhibitor (Z-DEVD-FMK; Calbiochem, Darmstadt, Germany) and neutralized with 10 μg/ml DKK1 monoclonal antibody (R & D Systems Inc.) and 10 μg/ml human IgG (CSL Ltd., Parkville, Australia) and then treated with 10 ng/ml IL-1β or 400 ng/ml DKK1. To investigate cell apoptosis, suspended and adhesive cells were pooled onto poly-lysine pre-coated glass slides then fixed with ice-cold methanol and then subjected to morphological observation and TUNEL staining.

CELL GROWTH

Cell proliferation was determined by Cell Proliferation Assay Kits (Roche Diagnostics, Mannheim, Germany) according to the manufacturer instructions. Briefly, cells (1×10^4 cells/well, 96-well plate) with and without IL-1β and DKK1 treatments were cultured for 48 h. Aliquots of 3-(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide were then added to each well. Formazan synthesized by the cultured cells was dissolved by 100 μl of 10% SDS−0.01 M HCl and the absorbance of the mixtures was colorimetrically measured at 550 nm (Amersham Biosciences Buckinghamshire, UK).

IMMUNOBLOTTING

Cytosolic and nuclear fractions of cell lysates were prepared for immunoblotting as previously described[23]. The designated proteins on the blots were probed by primary antibodies against caspase-3, cleaved caspase-3, cleaved poly (ADP-ribose) polymerase (PARP), β-catenin, phosphorylated-Ser473-Akt and actin (Cell Signaling Technology Inc., Beverly, MA) followed by horseradish peroxidase-conjugated IgG as the secondary antibody then visualized with chemiluminescence agents. For each blot in three repeated experiments, protein band intensity was quantified by scanning densitometry.

STATISTICAL ANALYSIS

All values were expressed as means ± standard deviations. The Wilcoxon test was used to evaluate differences between the clinical samples

of interest and their respective controls. *In vitro* experimental data were collected from at least three repeated experiments. Analysis of variance (ANOVA) was performed to evaluate differences between the samples of interest and their respective controls, and the Scheffe test was used for posterior comparison. The histological data were analyzed by general linear model followed by partial correlation test to determine the significant differences between treatments. A P-value of <0.05 was considered statistically significant.

Results

PROMOTION OF INFLAMMATION AND APOPTOSIS IN OA CARTILAGES

The two groups did not significantly differ in age ($P = 0.353$) or gender ($P = 0.102$). Real time RT-PCR results revealed significantly increased expressions of IL-1β [$P = 0.0491$; Fig. 1(A)], TNF-α [$P = 0.0085$; Fig. 1(B)], proapoptosis regulators Bad [$P = 0.004$; Fig. 1(C)], Bax [$P = 0.0266$; Fig. 1(D)] and caspase-3 mRNA [$P = 0.008$; Fig. 1(E)] in OA patients. However, expression of anti-apoptosis modulator Bcl2 mRNA was significantly lower in OA patients [$P < 0.0001$; Fig. 1(F)]. These findings indicate the inflammatory and apoptotic activities in OA cartilages.

ASSOCIATION OF DKK1 WITH INFLAMMATION AND APOPTOSIS IN OA CARTILAGES

Expression of DKK1 was examined for associations with OA. Quantitative RT-PCR results showed that OA cartilages had higher DKK1 mRNA expression than controls [$P = 0.0396$; Fig. 2(A)]. Moreover, DKK1 mRNA expression significantly correlated with expression of IL-1β [$r = 0.9993$, $P < 0.0001$; Fig. 2(B)], TNF-α [$r = 0.7365$, $P = 0.037$; Fig. 2(B)], Bad [$r = 0.9569$, $P < 0.0001$; Fig. 2(C)], and caspase-3 mRNA [$r = 0.7502$, $P = 0.031$; Fig. 2(D)].

INCREASED DKK1 EXPRESSION CORRELATES WITH CHONDROCYTE INFLAMMATION AND APOPTOSIS

Immunohistochemistry was examined in inflammatory cytokines, DKK1, cleaved caspase-3 and TUNEL staining in cartilage microenvironments. Cells with positive IL-1β, TNF-α and DKK1 displayed brown staining in the periphery and cytoplasm. Cells with positive cleaved caspase-3 expression and TUNEL staining displayed brown and red staining in the nucleus. Chondrocytes in OA cartilage exhibited intense DKK1, IL-1β, TNF-α, cleaved caspase-3

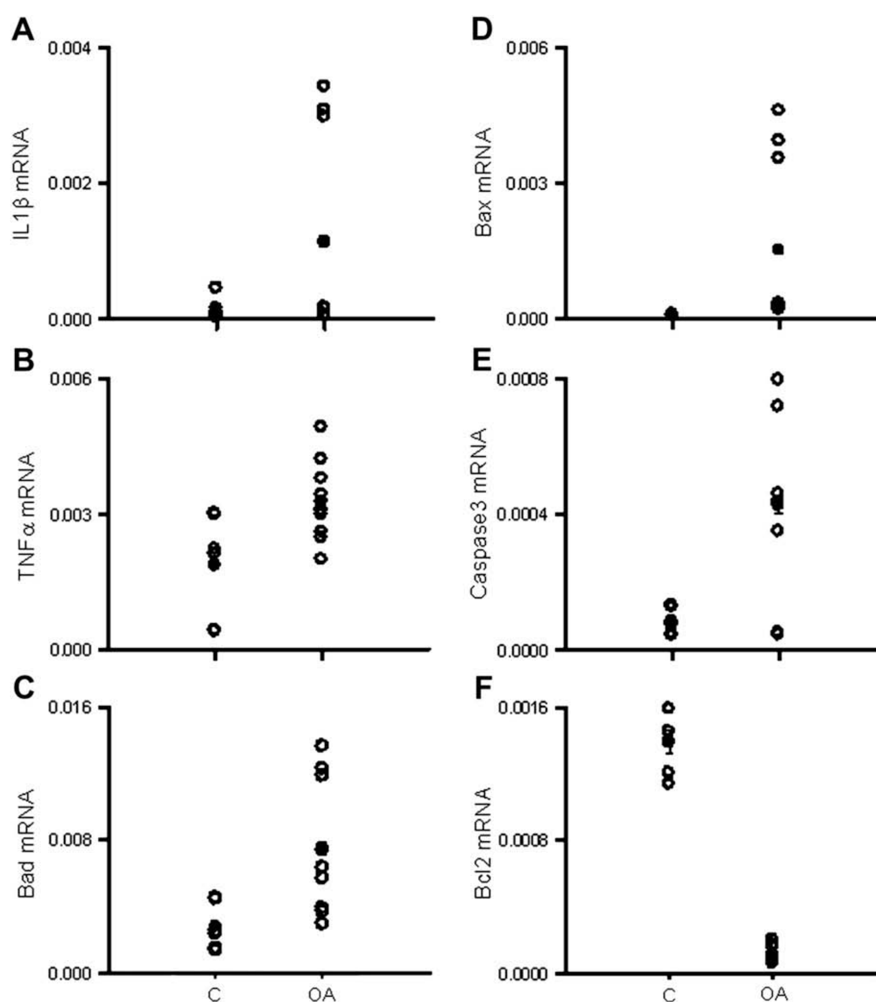

Fig. 1. Articular cartilage specimens from osteoarthritic joints had higher mRNA expression levels of (A) IL-1β, (B) TNF-α, (C) Bad, (D) Bax, and (E) caspase-3 and lower mRNA expression of (F) Bcl2 than those from femoral neck fracture patients. The mRNA expression was analyzed by real time RT-PCR. The relative abundance of the targeted gene was normalized to that of the housekeeping gene *18S* and was presented as $2^{(-\Delta Ct)}$, where $\Delta Ct = Ct_{target\ gene} - Ct_{18S}$.

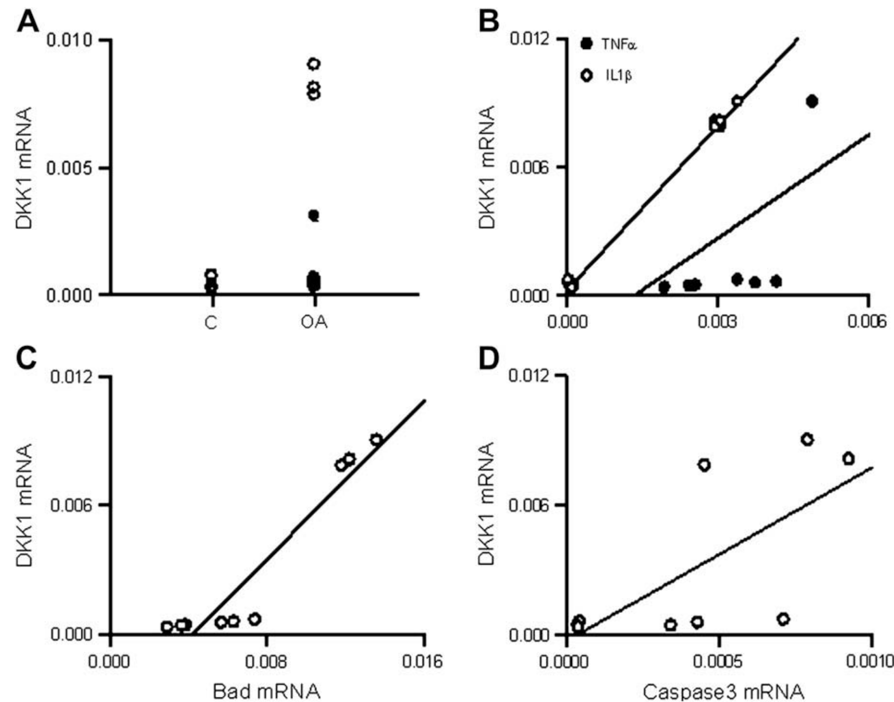

Fig. 2. The articular cartilage harvested from patients with osteoarthritic joint exhibited higher levels of (A) DKK1 mRNA expression than that harvested from patients with femoral neck fractures. The mRNA expression was analyzed by real time RT-PCR. The relative abundance of the targeted gene was normalized to that of the housekeeping gene *18S*. Increased DKK1 mRNA expression was correlated with increased mRNA expression of (B) IL-1β and TNF-α, (C) Bad and (D) caspase-3 in the articular cartilage of OA patients.

expressions and TUNEL staining [Fig. 3(A)]. Chondral cells in the control group displayed weak expressions of DKK1, IL-1β, TNF-α, cleaved caspase-3 and TUNEL staining [Fig. 3(A)]. Histomorphometric analyses showed that OA patients had significantly higher DKK1 expression associated with intense IL-1β, cleaved caspase-3, TNF-α immunoreactivity and TUNEL staining in chondrocytes [Fig. 3(B)]. The DKK1 immunostaining significantly correlated with IL-1β ($r = 0.468$, $P < 0.0001$), TNF-α ($r = 0.7201$, $P < 0.0001$), cleaved caspase-3 expression ($r = 0.7216$, $P < 0.0001$), and TUNEL staining ($r = 0.9983$, $P < 0.0001$).

DKK1 MEDIATES IL-1β INDUCTION OF APOPTOSIS PROGRAMS IN HUMAN FETAL CHONDROCYTES

Further experiments investigated whether DKK1 participates in inflammatory cytokine induction of chondrocyte apoptosis. Human fetal chondrocyte cultures were treated with recombinant human IL-1β. The IL-1β dose-dependently increased the number of apoptotic cells [Fig. 4(A)] and the expression of DKK1 mRNA in cell cultures [Fig. 4(B)]. Of the varying recombinant IL-1β concentrations, the 10 ng/ml IL-1β dose produced the greatest chondrocyte apoptosis and was used in subsequent experiments. Recombinant human DKK1 treatment increased chondrocyte apoptosis. The 400 ng/ml DKK1 treatment was the most potent treatment for promoting chondrocyte apoptosis and was used in the following experiments [Fig. 4(C)]. The DKK1 treatment time-dependently increased chondrocyte apoptosis [Fig. 4(D)]. Fluorescence microscopy revealed chondrocytes with fragmented chromatin by TUNEL staining (green fluorescence) and DAPI staining (blue fluorescence) in the nucleus. In both IL-1β and DKK1 groups, chondrocytes displayed intense fragmented chromatin formation. Few cells in the control group displayed fragmented chromatin [Fig. 4(E)].

Quantitative RT-PCR results indicated that IL-1β significantly promoted Bad [Fig. 5(A)], Bax [Fig. 5(B)] and caspase-3 mRNA expression [Fig. 5(C)], but inhibited Bcl2 mRNA expression [Fig. 5(D)]. However, the regulatory effects could be suppressed by DKK1 monoclonal antibody neutralization. Recombinant DKK1 treatment significantly promoted Bad, Bax and caspase-3 mRNA expression but reduced Bcl2 expression. Immunoglobulin treatment did not significantly alter the regulatory effects of IL-1β on Bad, Bax, caspase-3 or Bcl2 mRNA expression (Fig. 5). Moreover, IL-1β and DKK-1 treatment increased cleaved caspase-3 (corresponding to 17 kDa) and cleaved PARP (corresponding to 89 kDa) expressions [Fig. 6(A)], promoted cell apoptosis [Fig. 6(B)] and attenuated cell culture growth [Fig. 6(C)]. Caspase-3 inhibitor Z-DEVD-FMK treatment abrogated IL-1β- and DKK1-mediated caspase-3 and PARP cleavage but increased chondrocyte survival. DKK1 antibody neutralization moderated IL-1β-induced apoptosis as well as promoted cell proliferation. Immunoglobulin treatment did not significantly alter IL-1β-induced apoptotic activity in cell cultures (Fig. 6).

DKK1 MODULATES Akt AND β-CATENIN SIGNALING

Immunoblotting revealed that IL-1β treatment attenuated expressions of phosphorylated-Ser473-Akt and nuclear β-catenin in the chondrocyte cultures (Fig. 7). The DKK1 antibody neutralization increased nuclear β-catenin accumulation and phosphorylated Akt expression of chondrocytes exposed to IL-1β stress. Recombinant DKK1 treatment reduced nuclear β-catenin accumulation and Akt phosphorylation of chondrocyte cultures (Fig. 7).

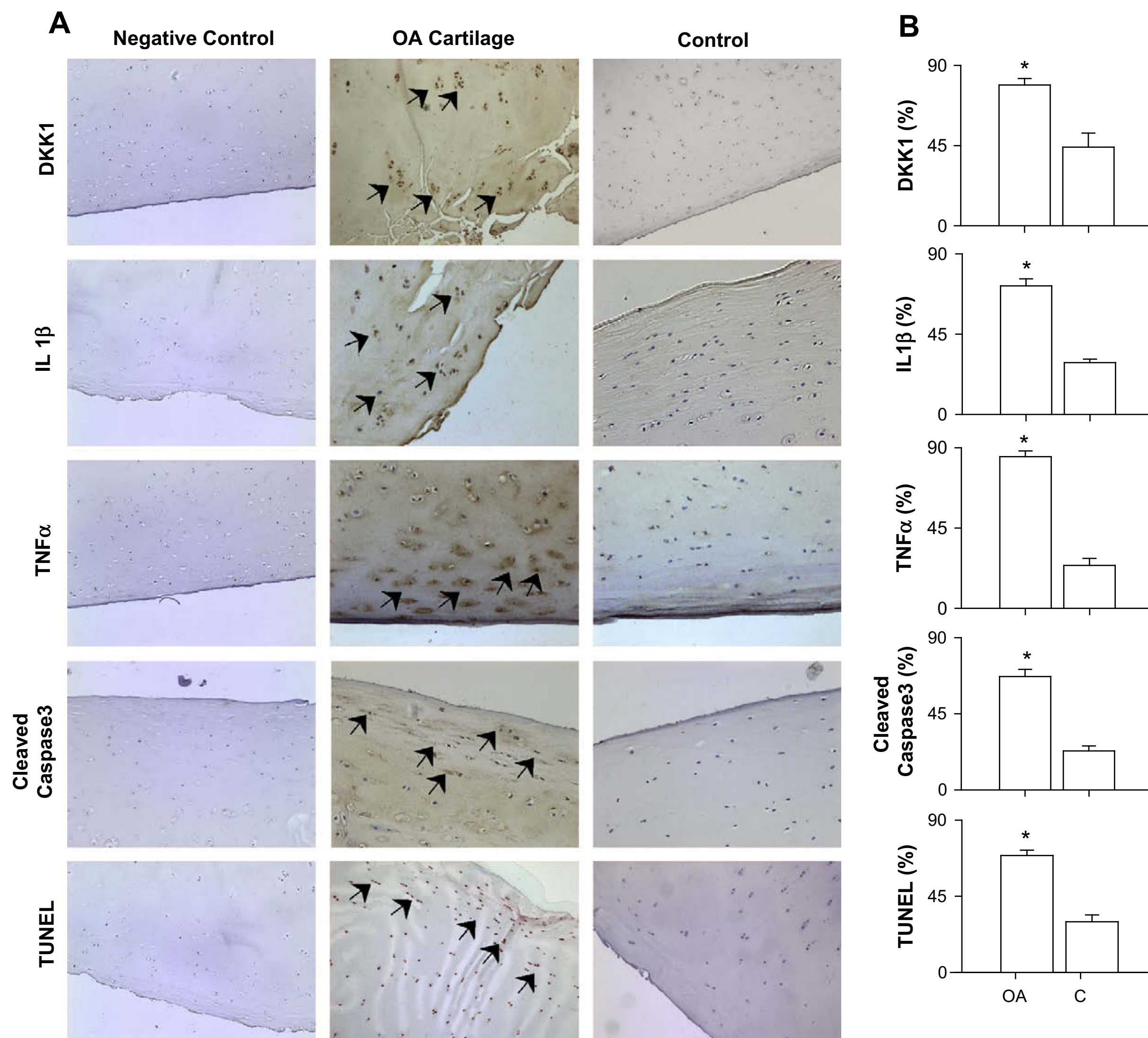

Fig. 3. Representative immunohistochemical photographs of (A) DKK1, IL-1β, TNF-α, cleaved caspase-3 and TUNEL staining in osteoarthritic cartilages and control samples. Chondrocytes in OA cartilages displayed intense expressions of DKK1, IL-1β, TNF-α and cleaved csapase-3 as well as TUNEL staining (arrows). (B) Histomorphometric analyses showed that OA patients had significantly higher DKK1, IL-1β, TNF-α, and cleaved caspase-3 expressions and TUNEL staining in chondrocytes. The "*" indicates differences between the OA and control groups, $P < 0.05$.

DKK1 MEDIATES IL-1β-INDUCED APOPTOSIS IN *EX VIVO* CHONDROCYTES FROM ADULT SUBJECTS

Further experiments investigated whether IL-1β or DKK1 induces apoptosis of primary human chondrocytes harvested from the three OA joint cartilage of adult subjects. The IL-1β and DKK1 treatments significantly increased mRNA expressions of Bad [Fig. 8(A)], Bax [Fig. 8(B)], caspase-3 [Fig. 8(C)] and apoptotic cell number in primary chondrocytes [Fig. 8(E)]. The IL-1β and DKK1 treatments significantly inhibited Bcl2 mRNA expression [Fig. 8(D)] and cell culture proliferation [Fig. 8(F)]. DKK1 monoclonal antibody neutralization and Z-DEVD-FMK treatment significantly abrogated apoptotic activity and reversed the growth of IL-1β- and DKK1-stressed primary chondrocyte cultures (Fig. 8). Caspase-3 inhibitor Z-DEVD-FMK abrogated IL-1β- and DKK1-mediated suppression of primary human chondrocytes survival [Fig. 8(E)] and cell proliferation [Fig. 8(F)]. Immunoglobulin treatment did not significantly alter apoptosis programs in IL-1β-stressed primary human chondrocyte cultures. TUNEL and DAPI staining revealed that cells displayed intense fragmented chromatin formation in IL-1β and DKK1 groups. Few chondrocytes displayed fragmented chromatin in the DKK1 antibody neutralization and caspase-3 inhibitor-treated groups (Fig. 9). Recombinant IL-1β and DKK1 also promoted apoptosis of chondrocytes isolated from joint cartilage in fracture femoral neck. Caspase-3 inhibitor pretreatment and DKK1 antibody neutralization attenuated the promoting effects of IL-1β and DKK1 on chondrocyte apoptosis (Supplementary data).

Discussion

Upregulated DKK1 expression correlated with increased inflammatory cytokine expression in OA joints. This Wnt

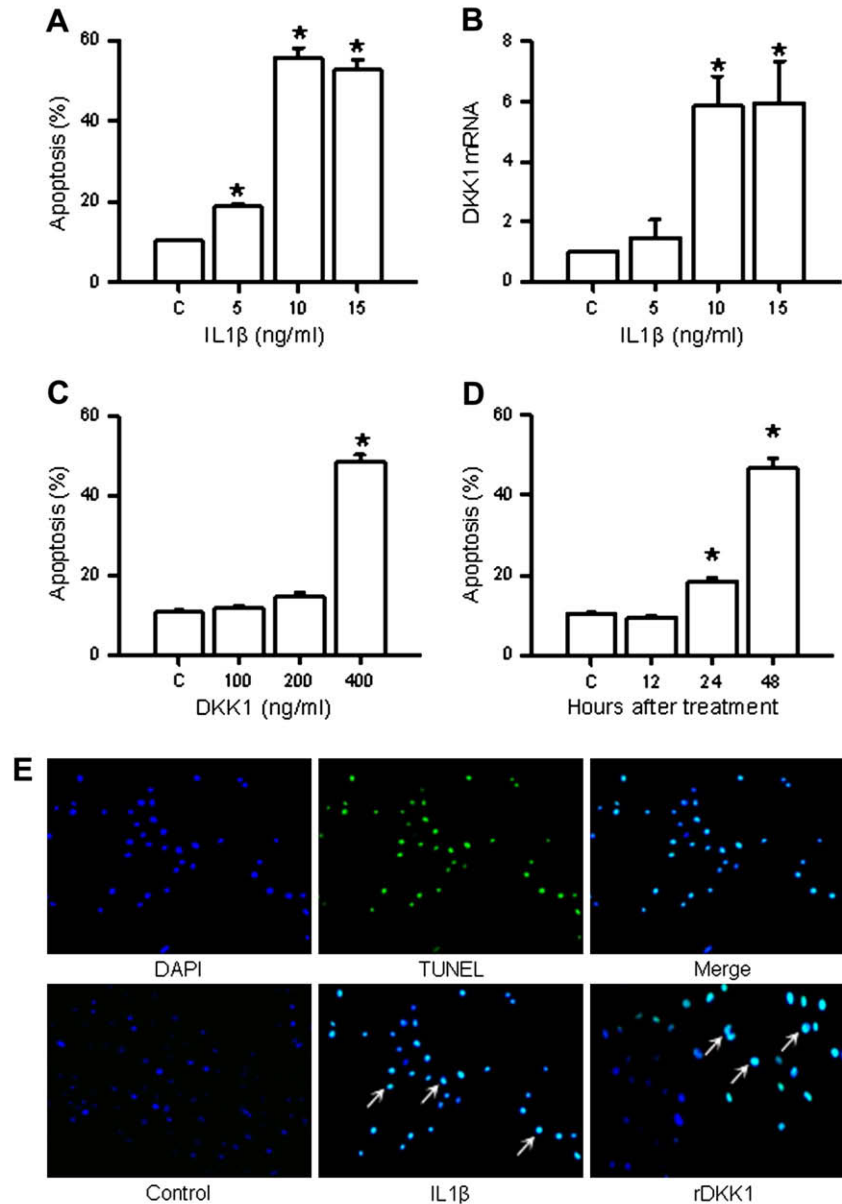

Fig. 4. Effects of recombinant human IL-1β and DKK1 treatments on apoptosis and DKK1 expression of chondrocyte cultures. The IL-1β treatment dose-dependently increased (A) the number of apoptotic cells and (B) DKK1 mRNA expression in chondrocyte cultures. (B) Recombinant human DKK1 treatment (C) dose- and (D) time-dependently increased the number of apoptotic chondrocytes. (E) Representative fluorescence staining of apoptotic chondrocytes. Fragmented chromatin in cell cultures was detected by TUNEL (green fluorescence) and DAPI (blue fluorescence) staining. Human fetal chondrocytes (1×10^5 cells/well; 6-well plate) with and without recombinant IL-1β and DKK1 treatments were cultured in DMEM with 10% fetal bovine serum for 48 h. The mRNA expressions were measured by quantitative RT-PCR and normalized to the housekeeping gene *18S*. The "*" indicates differences between the vehicle-treated, IL-1β-treated and DKK1-treated groups, $P < 0.05$.

inhibitor was linked to promotion of chondrocyte apoptosis in the OA cartilage. Growing evidence indicates that the Wnt pathway regulates cartilage integrity and joint development[24–26] and that upregulated Wnt antagonism action is linked to the pathogenesis of joint disorders[15,27]. However, the pathogenic role of the Wnt inhibitor DKK1 in the fate of chondral cells in OA joints has seldom been addressed. The current findings are, to our knowledge, the first indication that DKK1 mediates inflammatory cytokine suppression of Akt activation and nuclear β-catenin accumulation and subsequently induces chondrocyte apoptosis. These findings suggest that DKK1-mediated proapoptotic activity in chondrocytes contributes to osteoarthritic joint formation. Inflammation disturbance of DKK1/β-catenin signaling in chondrocytes provides a new molecular insight into the pathogenesis of diminished cartilage integrity associated with OA.

Chondrocytes in the OA cartilage intensely expressed IL-1β and TNF-α associated with OA. The mechanisms underlying intense inflammatory cytokine expression in OA joint microenvironments are unclear. Increased cartilage inflammation impairs extracellular matrix accumulation of chondral cells during the pathogenesis of joint disorders,

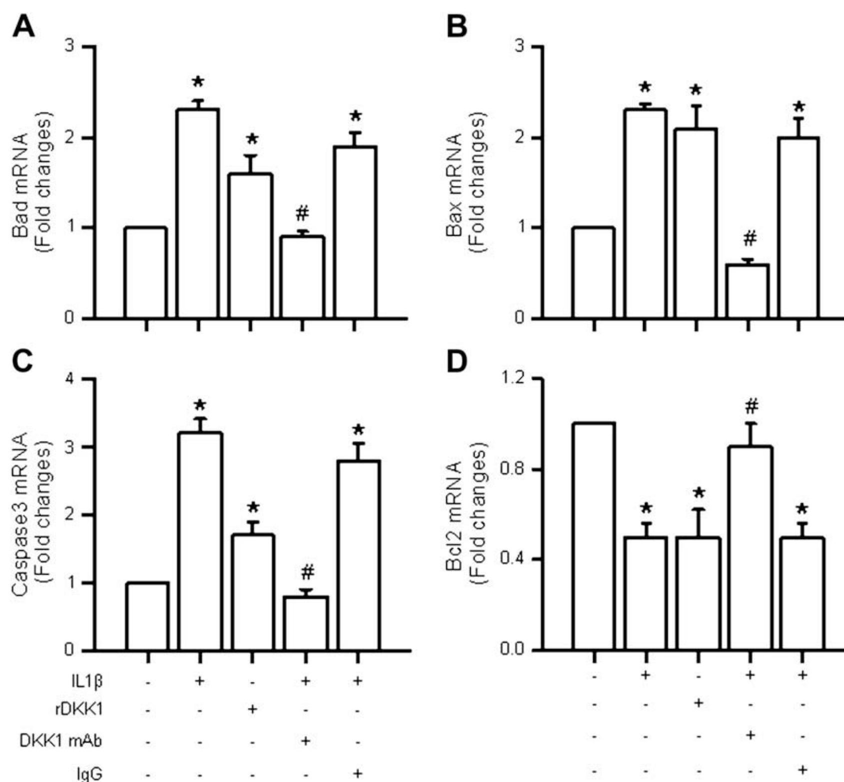

Fig. 5. Effects of recombinant IL-1β and DKK1 treatments on apoptotic activity of chondrocyte cultures. The IL-1β treatment increased (A) Bad, (B) Bax and (C) caspase-3 mRNA expressions but inhibited (D) Bcl2 mRNA expression of cell cultures, which was abrogated by DKK1 monoclonal antibody neutralization. Recombinant DKK1 treatment significantly regulated proapoptosis and anti-apoptosis gene expression in chondrocytes. Human fetal chondrocytes (1×10^5 cells/well; 6-well plate) with and without recombinant 10 ng/ml IL-1β, 400 ng/ml DKK1, 10 μg/ml DKK1 monoclonal antibody and 10 μg/ml immunoglobulin treatments were cultured in DMEM with 10% fetal bovine serum for 48 h. The mRNA expressions were measured by quantitative RT-PCR and normalized to the housekeeping gene *18S* and presented as $2^{-\Delta\Delta Ct}$. The "*" and "#" indicate differences between the vehicle-treated, IL-1β-treated and DKK1-treated groups, respectively, $P < 0.05$.

apparently as a biological reaction to deteriorating cartilage function[6,28]. In the current study, promotion of proapoptosis regulators Bad, Bax and caspase-3 expressions as well as attenuation of anti-apoptosis modulator Bcl2 expression and chondrocyte apoptosis coincided with increased inflammatory cytokine expression in OA cartilage, suggesting that inflammation-mediated chondrocyte apoptosis contributes to the disruption of cartilage integrity. These findings agree with previous studies that demonstrate the upregulation of IL-1β expression in OA articular cartilage[29]. Diminished chondrocyte survival due to inflammation is a proposed pathogenic cause of OA. Previous studies demonstrate that Bcl2 expression is higher in advanced OA lesions than in non-OA sites or in sites with minimal OA damage[30]. However, articular cartilage in normal subjects is known to have higher Bcl2 immunostaining than that in OA patients[31]. The Bcl2 gene expression in advanced OA cartilage is lower than that in cartilage with minimal OA damage[32]. The current study revealed reduced Bcl2 mRNA expression in cartilage tissue harvested from patients with end-stage OA. We speculate that cartilage with varying severity of OA differ in Bcl2 expression.

While Wnt antagonist sFRP is known to inhibit chondrocyte differentiation and growth, growing evidence demonstrates that reactive oxygen radicals, endothelin-1, glucocorticoids, tau and thalidomide participate in DKK1-mediated tissue remodeling and tumoriogenesis[33–37] and that DKK1 inhibits thyroid hormone-mediated chondrocyte differentiation of growth plate[38]. However, the biological role of DKK1 in the development of OA has not been characterized. This study provides novel evidence that increased DKK1 expression correlates with inflammatory cytokine expression and chondrocyte apoptosis in OA cartilage. The DKK1 is a potent apoptosis-promoting factor in modulating the development of skeletal tissue and several cell types[39–41]. Moreover, chondrocyte cultures respond to IL-1β stress by increasing DKK1 expression and apoptosis programs. The phenomena observed in the *in vitro* model were consistent with those detected in both *in vivo* and *ex vivo* primary chondrocyte cultures. We suggest that, in OA pathogenesis, DKK1 is at least one proapoptotic factor that mediates inflammatory cytokine-induced caspase-3-dependent chondral cell apoptosis. This is based on the observation that DKK1 antibody neutralization abrogates the promoting effect of IL-1β on proapoptotic gene expression and *in vitro* programmed death of chondrocytes. Recombinant DKK1 treatment alone increases apoptosis in chondrocyte cultures. To our knowledge, this is the first report that disturbed DKK1 expression modulates inflammation induction of chondrocyte apoptosis in OA articular cartilage.

The IL-1β treatment attenuated Akt phosphorylation and nuclear β-catenin accumulation but was abrogated by DKK1 monoclonal antibody neutralization. The role of β-catenin signaling in controlling chondrocyte function and cartilage structure is controversial. The β-catenin signaling is

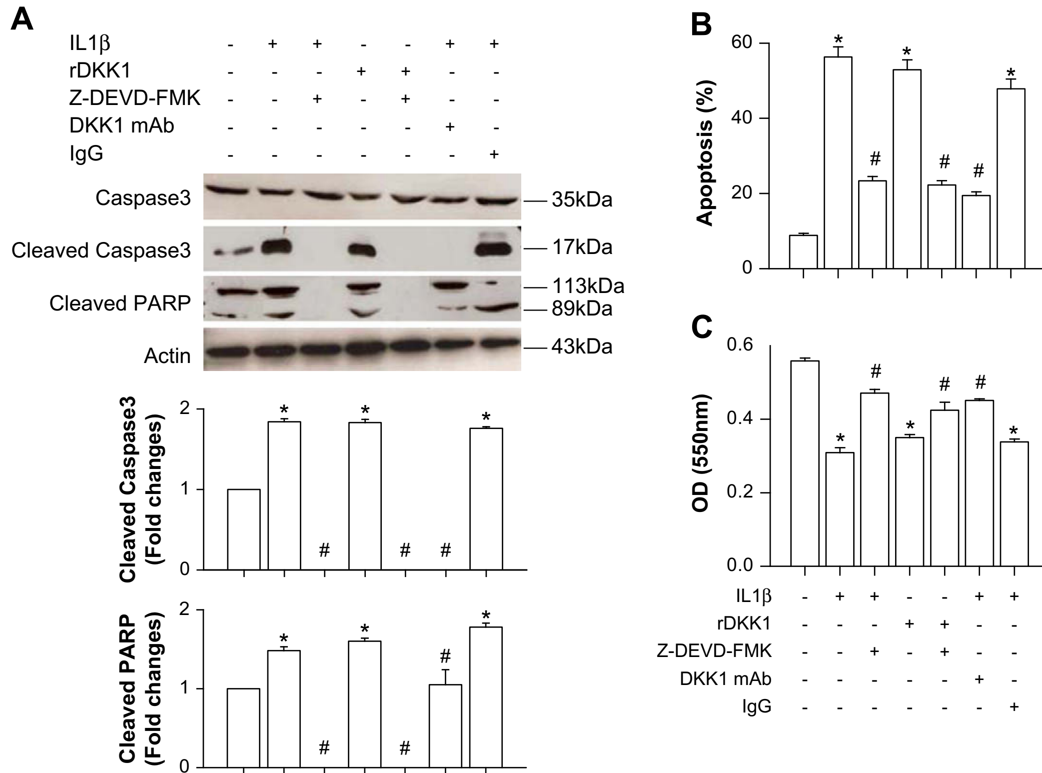

Fig. 6. Effects of IL-1β and DKK1 on chondrocyte survival. The IL-1β and DKK1 treatments (A) increased cleaved caspase-3 and cleaved PARP expressions, (B) promoted apoptotic cell number and (C) inhibited chondrocyte growth. Caspase-3 inhibitor and DKK1 monoclonal antibody neutralization attenuated cleaved caspase-3 and cleaved PARP expressions and increased survival of cell cultures. Human fetal chondrocytes (1×10^5 cells/well; 6-well plate) with and without recombinant 10 ng/ml IL-1β, 400 ng/ml DKK1, 10 μg/ml DKK1 monoclonal antibody, 10 μg/ml immunoglobulin and 10 μM caspase-3 inhibitor Z-DEVD-FMK treatments were cultured in DMEM with 10% fetal bovine serum for 48 h. The "*" and "#" indicate differences between the vehicle-treated, IL-1β-treated and DKK1-treated groups, respectively, $P < 0.05$.

reportedly an essential Wnt signaling component for regulating chondrogenic cell growth[42] and osteogenic cell survival[43]. Increased β-catenin suppresses chondrocyte differentiation[44] and is associated with the IL-1β-mediated de-differentiation of arthritic chondrocytes[45]. The DKK1 disturbs β-catenin signaling-dependent tissue regeneration activity[46]. In this study, recombinant DKK1 inhibited nuclear β-catenin accumulation and Akt phosphorylation,

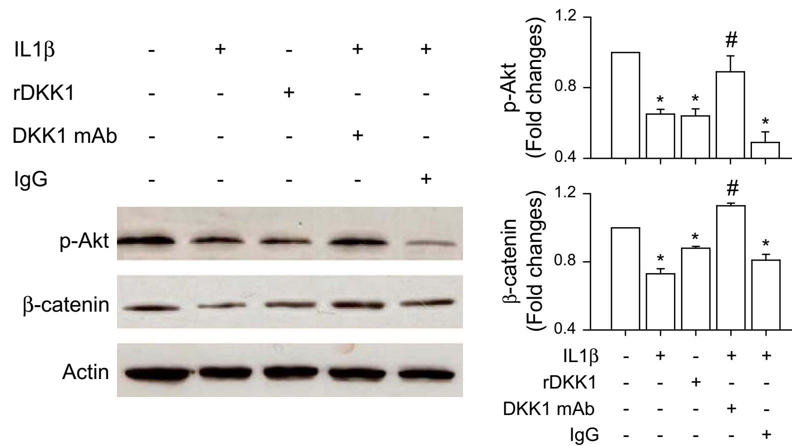

Fig. 7. Representative immunoblotting of nuclear β-catenin and phosphorylated-Ser473-Akt in chondrocytes exposed to IL-1β and recombinant DKK1 stresses, DKK1 antibody neutralization and immunoglobulin G. The IL-1β inhibited nuclear β-catenin accumulation and phosphorylated-Ser473-Akt expression. Recombinant DKK1 treatment reduced nuclear β-catenin accumulation and phosphorylated-Ser473-Akt expression in chondrocyte cultures. DKK1 antibody neutralization abrogated IL-1β-suppression of nuclear β-catenin and phsphorylated-Ser473-Akt expressions of chondrocytes. Actin on the blots showed equal loading and transfer for all lanes. The "*" and "#" indicate differences between the vehicle-treated, IL-1β-treated and DKK1-treated groups, respectively, $P < 0.05$.

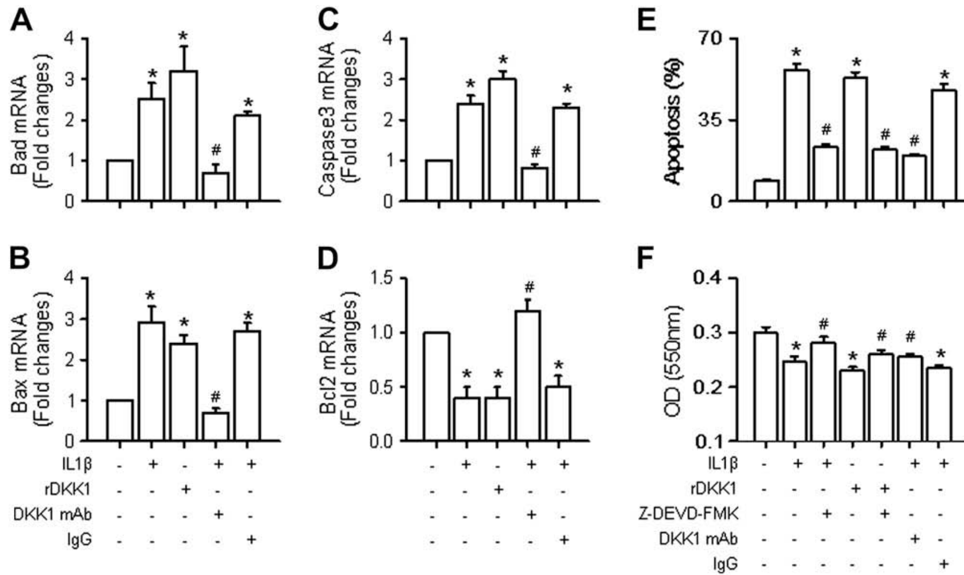

Fig. 8. Effects of IL-1β and DKK1 treatments on apoptosis in *ex vivo* primary human chondrocyte cultures. The IL-1β and DKK1 treatments increased (A) Bad, (B) Bax and (C) caspase-3 mRNA expressions and (E) apoptotic cell number. The IL-1β and DKK1 treatments inhibited (D) Bcl2 mRNA expression and (F) chondrocyte proliferation. DKK1 monoclonal antibody neutralization and caspase-3 inhibitor attenuated apoptosis activity and increased growth of IL-1β- and DKK1-treated chondrocytes. Primary human chondrocyte cultures were harvested from OA knee cartilage in adult subjects. Cells (1×10^5 cells/well; 6-well plate) with and without recombinant 10 ng/ml IL-1β, 400 ng/ml DKK1, 10 μg/ml DKK1 monoclonal antibody, 10 μg/ml immunoglobulin and 10 μM caspase-3 inhibitor Z-DEVD-FMK treatments were cultured in DMEM/Ham's F12 with 10% fetal bovine serum for 48 h. The mRNA expressions were measured by quantitative RT-PCR and normalized to the housekeeping gene *18S* and presented as $2^{-\Delta\Delta Ct}$. The "*" and "#" indicate differences between the vehicle-treated, IL-1β-treated and DKK1-treated groups, respectively, $P < 0.05$.

suggesting that β-catenin and Akt are essential for chondrocytes survival after exposed to IL-1β and DKK1 stress.

Previous studies indicate that IL-1β treatment does not induce apoptosis in immortalized human juvenile chondrocytes[47] and normal human articular chondrocytes[48]. IL-1β and TNF-α co-treated with actionmycin D[6], oxygen radical scavenger[49] and kinase inhibitors[50] induce apoptosis of human chondrocyte cultures from OA cartilage. The current study demonstrated that IL-1β promotes expression of proapoptosis regulators as well as apoptosis of human fetal chondrocyte cultures and primary chondrocytes in cartilage harvested from adult subjects. The results of this study agree previous reports that IL-1β suppresses viability and induces apoptotic signaling in human chondrocytes harvested from articular cartilage[51,52] and OA cartilage[53,54]. In this study, the observation of increased expression of apoptosis-promoting factor DKK1, caspase-3 and PARP cleavage and suppression of anti-apoptosis regulator and cell proliferation also support the debilitating effect of IL-1β on chondrocyte cultures. We speculate that the discrepant deleterious effects of IL-1β on chondrocyte apoptosis may be due to varying concentration and duration of IL-1β treatment.

In addition to cartilage degradation, synovitis and subchondral bone destruction with excess remodeling, sclerosis and osteophytes reportedly contribute to OA

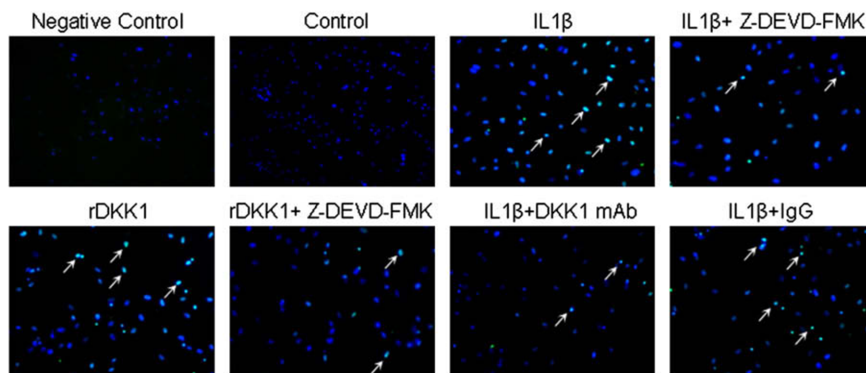

Fig. 9. Representative fluorescence staining of apoptotic primary human chondrocytes. Fragmented chromatin in cell cultures was detected by TUNEL (green fluorescence) and DAPI (blue fluorescence) staining. The IL-1β and DKK1 treatments promoted chondrocyte apoptosis, which were alleviated by DKK1 monoclonal antibody neutralization and capase-3 inhibitor Z-DEVD-FMK treatment. Recombinant DKK1 treatment increased chondrocyte apoptosis.

pathogenesis[55]. The findings of this study do not exclude the possibility that DKK1 alters the cell fate or deteriorates the microstructures of these tissues. Inflammatory cytokines may directly or indirectly alter DKK1 expression. An acknowledged limitation of this study is that the articular cartilage specimens were not compared with those from healthy subjects. However, our observations confirm the correlation between increased DKK1 expression and the incidence of joint OA. This study provides novel evidence of a pathomechanism in which Wnt inhibitor DKK1 has a potent cartilage-deleterious role in the pathogenesis of OA. Further studies may examine whether attenuating DKK1 action prevents cartilage deterioration in OA.

Conflict of interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article. Funds were received in partial support for the research presented in this article. The funding sources were from National Science Council and Chang Gung Memorial Hospital, Taiwan.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.joca.2008.12.008.

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