CTHRC1 mediates IL-1β-induced apoptosis in chondrocytes via JNK1/2 signaling

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Abstract. Osteoarthritis (OA), also known as degenerative joint disease or degenerative arthritis, is characterized by chondrocyte apoptosis. The aim of the present study was to investigate the effects of collagen triple helix repeat containing 1 (CTHRC1) and the c-Jun N-terminal kinase (JNK) 1/2 inhibitor SP600125 on rat chondrocytes cultured in vitro with interleukin (IL)-1β. Chondrocytes were treated with different doses of IL-1β and cell viability and CTHRC1 expression were assessed using Cell Counting Kit-8 and western blot assays, respectively. In separate experiments, chondrocytes were treated with CTHRC1-expressing constructs (pLVX-Puro-CTHRC1) and/or SP600125, or IL-1β with either CTHRC1 short hairpin (sh)RNA constructs (shRNA-CTHRC1) or SP600125. The expression of CTHRC1, B-cell lymphoma (Bcl)-2, Bcl-2-associated X protein (Bax), cleaved caspase-3, poly ADP ribose polymerase (PARP)-1 and matrix metalloproteinase (MMP)-13 was measured using reverse transcription-quantitative polymerase chain reaction and western blotting assays. A Cell Counting Kit-8 assay was performed to examine cell viability. Annexin V/propidium iodide staining and flow cytometry assays were used to detect chondrocyte apoptosis. The expression of JNK1/2 and phosphorylated JNK1/2 was measured using western blotting. CTHRC1 was highly expressed in patients with OA compared with normal controls. IL-1β treatment (5, 10 and 20 ng/ml) increased the protein expression of CTHRC1 in a dose-dependent manner and decreased the viability of chondrocytes in a time-dependent manner. pLVX-Puro-CTHRC1 mimics the effect of IL-1β on chondrocyte apoptosis and JNK1/2 activity, and this is reversed by SP600125 treatment. However, transfection with shRNA-CTHRC1 or treatment with SP600125 inhibited IL-1β-induced cell apoptosis and JNK1/2 activation. These results indicate that CTHRC1 downregulation may protect chondrocytes from IL-1β-induced apoptosis by inactivating the JNK1/2 pathway.

Introduction

Osteoarthritis (OA) is a prevalent degenerative joint disease associated with aging, obesity and trauma and is the most common form of arthritis characterized by the progressive destruction of articular cartilage, with >270 million cases reported worldwide (1). Approximately 80% of OA cases diagnosed by radiography occur in patients over 65 years old (2). OA typically causes joint instability, pain, loss of function, stiffness and mobility difficulties (3), altogether leading to a deterioration in quality of life and increasing the cost of health care for the aging population (4). The etiology and pathogenesis of OA are not well understood due to a combination of various risk factors and initiating mechanisms (5). Furthermore, a significant and positive correlation between the degeneration of articular cartilage (6), which is the root cause of OA, and bioactive compounds, including pro-inflammatory cytokines and a dipokines (7), has been demonstrated.

Although the pathogenesis of OA has multiple underlying mechanisms that are not well understood, it has been reported that decreased chondrocyte proliferation and apoptosis dysregulation are significantly associated with OA pathogenesis and are observed in OA cartilage more frequently compared with normal cartilage (8). Furthermore, chondrocyte apoptosis is positively correlated with cartilage degradation during the development and progression of OA (9). Chondrocytes are required to maintain cartilage structure and function via the production of extracellular matrix components (10), which are responsible for maintaining the cartilaginous matrix. Consequently, the coordinated regulation of chondrocyte proliferation and apoptosis is of great importance in cartilage function and cartilage injury repair due to OA. Pro-inflammatory cytokines, including interleukin-1β (IL-1β), are important for dysregulated chondrocyte apoptosis and, together with matrix metalloproteinases (MMPs), trigger a vigorous pro-inflammatory response (11,12). In addition to MMP-1, MMP-13 is also important in the pathological process of OA, inducing matrix degradation, further chondrocyte senescence and aging changes (13). However, cellular responses to upregulation and downregulation of IL-1β do not dominate the overall gene expression signature.
in osteoarthritic chondrocytes (14). Therefore, the mechanism of IL-1β regulation in osteoarthritic cartilage degeneration remains unclear.

A previous study reported that the expression of collagen triple helix repeat containing 1 (CTHRC1) was upregulated in OA (15), indicating a central role of CTHRC1 in OA progression. However, the molecular mechanisms of CTHRC1 associated with the development and progression of OA are not well understood. The aim of the present study was to investigate the function of CTHRC1 in an IL-1β-induced OA model in rat chondrocytes in vitro. The results suggest that CTHRC1 downregulation inhibits IL-1β-induced chondrocyte apoptosis via inactivating JNK1/2 signaling.

Materials and methods

Tissue specimens. OA joint fluid samples (n=50) were collected from patients (67.9±7.2 years; male: female, 11:39) with OA who underwent knee arthroplasty at The First Affiliated Hospital of Anhui Medical University (Hefei, China) between June 2012 and April 2016. Human normal joint fluid samples were collected from 30 patients (62.8±11.2 years old; male: female, 1:4) with trauma and no history of OA or other joint diseases at The First Affiliated Hospital of Anhui Medical University. All patients who presented with obvious joint injury or with generalized OA were excluded from the study. The present study was approved by the Ethics Committee of Anhui Medical University. Written informed consent was obtained from all participants of this study and all investigations were performed in accordance with the Declaration of Helsinki. All patients agreed to the use of their samples in scientific research.

Cell culture. Articular chondrocytes were harvested from 20 male 4-week-old Sprague Dawley rats (250-300 g; Shanghai BK Experimental Animal Center, Shanghai, China), which were provided with free access to food and water and kept under a 12 h light/dark cycle at a constant temperature of 25°C in a humidified atmosphere containing 5% CO₂. Chondrocytes were treated with 75% ethanol for 10 min, washed with PBS and digested with 4 ml collagen II (EMD Millipore, Billerica, MA, USA) at 37°C for 5 h. The chondrocytes were collected by centrifugation at 400 x g for 5 min at 37°C and resuspended with Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 15% fetal bovine serum (FBS; HyClone; Thermo Fisher Scientific, Inc., Logan, UT, USA) and cultured at 37°C in an atmosphere containing 5% CO₂. Immunohistochemistry was performed when cells reached 50-60% confluence and cells were subsequently cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in an atmosphere containing 5% CO₂. The present study was approved by the Ethics Committee of Anhui Medical University.

Construction of pLVX-Puro-CTHRC1 lentiviral vector and transfection. cDNA encoding CTHRC1 was obtained using GENEWIZ (Suzhou, China) and cloned into pLVX-Puro to generate a CTHRC1 expression vector. The forward primer was 5'-GGG ATC CTG GGG GGG ATT ATG-3' and the reverse primer was 5'-GCG ATC CTT TGG AGG GGA T-3'. pcDNA encoding human CTHRC1 was obtained using KAPA203 (Kaiji Biological Engineering Materials Co., Ltd., Nanjing, China) was used according to the manufacturer's protocol to examine the activity of caspase-3. Chondrocytes (5x10⁶ cells/ml) were centrifuged at 1,000 x g for 5 min at 4°C. Pellets were fixed overnight in 70% cold ethanol. Following fixation, cells were washed twice with PBS and incubated in PBS containing RNase (1 mg/ml) for 10 min at room temperature. Finally, samples were mixed with 195 µl Annexin V FITC and 5 µl propidium iodide and incubated with 10 µl CCK-8 solution at 37°C for 1 h in an atmosphere containing 5% CO₂. Cell proliferation was calculated using a microplate reader (ELX 800; Bio-Tek Instruments, Inc., Winooski, VT, USA) at a wavelength of 450 nm.
caspase-3 substrate and incubated at 37°C for 4 h in the dark. Samples were read at 405 nm using a Multiskan EX microplate reader (Labsystems, Helsinki, Finland).

**RT-quantitative PCR.** Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized from isolated RNA using a PrimeScript RT reagents kit (Takara, Dalian, China). The conditions were as follows: 37°C for 60 min, 85°C for 5 min and 4°C for 5 min. PCR was performed using a DyNAseq Flash SYBR Green qPCR kit (Finnzymes Oy; Thermo Fisher Scientific, Inc.). The PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 45 sec, a final extension step of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. Data collection was performed using an Applied Biosystems 7300 Fast Real-Time PCR System (Thermo Fisher Scientific, Inc.) and relative quantification of gene expression was calculated using the 2-ΔΔcq method (16) with GAPDH as a reference gene. To compare relative mRNA expression levels, the expression of CTHRc1, B-cell lymphoma (Bcl)-2, Bcl-2-associated X protein (Bax), cleaved caspase-3, poly ADP ribose polymerase (PARP)-1 and matrix metalloproteinase (MMP)-13 were given as ratios to GAPDH. The primers were designed using Primer Express software (v3.0.1; Thermo Fisher Scientific, Inc.) and were as follows: CTHRc1, forward 5'-CTCGCCTTCCGCTCAAATG-3' and reverse 5'-GCACCA ATCCCTTCCAGC-3'; MMP-13, forward 5'-CAGACGAGCA AGAATAAGAC-3' and reverse 5'-CAACATAAGCAGGT GTAC-3'; Bcl-2, forward 5'-GGGTGCTGGCTTTGTTGGAAC-3' and reverse 5'-GTGGTGTGGTGGTGGTGGTGGTGG-3'; Bax, forward 5'-GGGACGATCCACCAAGAG-3' and reverse 5'-CTG CCACACGGAAGAC-3'; caspase-3, forward 5'-GGCC ATCTCCTGTGATTGG-3' and reverse 5'-CTGAGCCTACACTC CTTAC-3' and reverse 5'-ATGAGCCCTCTACAGATG-3'.

**Western blotting.** Total protein was extracted in lysis buffer supplemented with protease inhibitors (Beyotime Institute of Biotechnology). A total of 15 µl/lane protein was separated by 10-15% SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies: Anti-CTHRc1 (1:1,000 dilution; cat. no. ab185230; Santa Cruz Biotechnology, Inc.) and anti-MMP-13 (1:3,000 dilution; cat. no. ab39012; Abcam), primary rat chondrocytes were incubated with 3% H2O2 for 10 min and washed three times with 0.02 M PBS. The sections were stained with diaminobenzidine for 5 min at room temperature, counterstained with hematoxylin for 3 min at room temperature, and washed in water. For the negative controls, the primary antibody was omitted. Images were captured using a light microscope (Olympus Corporation, Tokyo, Japan; magnification, x200).

**Immunohistochemistry.** Cells were washed with 0.02 M PBS, fixed with 4% methanol for 30 min at room temperature, incubated with 3% H2O2 for 10 min and washed three times with 0.02 M PBS. Slides were incubated with anti-Collagen II (cat. no. ab34712; 1:200 dilution; Abcam) or anti-Sry-type high mobility group-box (SOX)9 (cat. no. ab185230; 1:200 dilution; Abcam) antibody at 4°C overnight and subsequently washed three times with 0.02 M PBS. The slides were stained with horseradish peroxidase-labeled goat anti-rat immunoglobulin G (cat. no. D-3004; 1:500 dilution; Shanghai Long Island Biotec Co., Ltd., Shanghai, China) for 30 min at 37°C and washed three times in PBS for 3 min each time. Subsequently, the sections were stained with diaminobenzidine for 5 min at room temperature, counterstained with hematoxylin for 3 min at room temperature and washed in water. Negative controls, the primary antibody was omitted. Images were captured using a light microscope (Olympus Corporation, Tokyo, Japan; magnification, x200).

**ELISA.** IL-1β and CTHRc1 expression in the joint fluid of patients with OA was determined using c THRc1 (cat. no. csb-EL006162HU; Cusabio Biotech Co., Ltd., College Park, MD, USA) and IL-1β (cat. no. 583311; Cayman Chemical Company, Ann Arbor, MI, USA) ELISA kits according to the manufacturer's protocol.

**Statistical analysis.** Results are presented as the mean ± standard deviation. All data were analyzed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Comparisons were made using t-test, analysis of variance and post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**IL-1β and CTHRc1 are upregulated in patients with OA.** Joint fluid from patients with OA was assessed using ELISA kits. The results demonstrated that the levels of IL-1β and CTHRc1 were significantly higher in the joint fluid of patients with OA compared with the normal controls, with IL-1β and CTHRc1 expression 65.7 and 113.6% higher, respectively (P<0.01; Fig. 1).

**CTHRc1 expression is increased in IL-1β-induced rat chondrocytes.** To investigate the role of CTHRc1 in osteoarthritic chondrocytes in vitro, primary rat chondrocytes were collected and chondrocyte-associated genes were assessed.
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using immunohistochemistry. SOX9 is a member of the Sox gene family, which are predominantly expressed in cartilage and activate Collagen II (17). Immunohistochemical staining demonstrated that Collagen II and SOX9 were highly expressed in normal primary cultured chondrocytes (Fig. 2A), which was indicative of a well-established chondrocyte system.
The exact cause of OA is not known; however, the degradation of extracellular matrix components is associated with elevated levels of the pro-inflammatory cytokine IL-1β (18). In the present study, IL-1β was introduced in rat chondrocytes to establish an in vitro osteoarthritis model. cTHRc1 protein expression was increased in chondrocytes in a dose-dependent manner in response to IL-1β (5, 10 and 20 ng/ml; P<0.01; Fig. 2B). Furthermore, chondrocyte proliferation was suppressed by IL-1β in a dose-dependent manner (P<0.01; Fig. 2C).

Figure 4. CTHRC1 upregulation induces chondrocyte apoptosis. (A and B) Chondrocyte apoptosis was assessed by flow cytometry analysis. (C) Changes in caspase-3 activity were investigated using spectrophotometry. (D and E) The expression of Bcl-2, Bax, cleaved caspase-3, PARP-1 and MMP-13 was studied using western blotting. **P<0.01 vs. vector and ##P<0.01 vs. pLVX-Puro-CTHRC1. CTHRC1, collagen triple helix repeat containing 1; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; PARP, poly ADP ribose polymerase; MMP, matrix metalloproteinase.

The exact cause of OA is not known; however, the degradation of extracellular matrix components is associated with elevated levels of the pro-inflammatory cytokine IL-1β (18). In the present study, IL-1β was introduced in rat chondrocytes to establish an in vitro osteoarthritis model. CTHRC1 protein expression was increased in chondrocytes in a dose-dependent manner in response to IL-1β (5, 10 and 20 ng/ml; P<0.01; Fig. 2B). Furthermore, chondrocyte proliferation was suppressed by IL-1β in a dose-dependent manner (P<0.01; Fig. 2C).

CTHRC1 upregulation activates the JNK1/2 pathway in chondrocytes. The pLVX-Puro-CTHRC1 vector was constructed to and transfected into chondrocytes to induce overexpression of CTHRC1. Levels of CTHRC1 mRNA and protein were studied using RT-qPCR and western blot analysis, respectively. The expression of CTHRC1 mRNA and protein was significantly increased in chondrocytes transfected with the pLVX-Puro-CTHRC1 vector compared with chondrocytes transfected with the empty pLVX-Puro vector (P<0.01; Fig. 3A-C). Furthermore, CTHRC1
upregulation significantly activated JNK1/2, and this activation was markedly reduced by the inhibitor, SP600125 (both P<0.01; Fig. 3D and E).

**CTHRC1 upregulation induces chondrocyte apoptosis.** Following transfection with pLVX-Puro-CTHRC1 for 24 h, the percentage of apoptotic cells and caspase-3 activity were significantly increased in chondrocytes (P<0.01; Fig. 4A-C); however, apoptosis and caspase-3 activity were significantly decreased following SP600125 treatment (P<0.01; Fig. 4B and C). The expression of MMP-13, Bcl-2, Bax, PARP-1 and cleaved caspase-3 was also measured using western blotting (Fig. 4D). The results revealed that CTHRC1 upregulation significantly increased the expression of MMP-13, Bax, PARP-1 and cleaved caspase-3 (P<0.01; Fig. 4E), whereas it significantly decreased Bcl-2 expression (P<0.01; Fig. 4E) compared with cells transfected with the empty vector. However, SP600125 treatment significantly decreased the expression of MMP-13, Bax, PARP-1 and cleaved caspase-3 and increased Bcl-2 expression in chondrocytes with pLVX-Puro-CTHRC1 transfection (P<0.01; Fig. 4E), suggesting that JNK1/2 signaling is associated with the mechanism of CTHRC1 upregulation in chondrocyte apoptosis.

**CTHRC1 downregulation suppresses IL-1β-induced apoptosis of chondrocytes.** To investigate the role of CTHRC1 downregulation in chondrocytes apoptosis, apoptotic cells were assessed using flow cytometry following treatment with 10 ng/ml of IL-1β for 24 h. The percentage of apoptotic cells was significantly increased compared with the control group (P<0.01; Fig. 6A and B). However, transfection with pLKO.1-CTHRC1-shRNA significantly attenuated this increase (P<0.05; Fig. 6A and B). To further explore the effect of JNK1/2 signaling in CTHRC1 downregulation-mediated protection against IL-1β-induced chondrocyte apoptosis, the JNK1/2 inhibitor SP600125 (10 µM) was added 30 min prior to IL-1β. SP600125 also markedly inhibited chondrocyte apoptosis in response to IL-1β (Fig. 6A and B; P<0.01 vs. IL-1β treatment alone).

To explore the role of CTHRC1 downregulation-mediated protection against IL-1β-induced chondrocyte apoptosis,
the expressions of MMP-13, Bcl-2, Bax and cleaved caspase-3 were detected via RT-qPCR and western blotting. Compared with control cells, expression of Bcl-2 was significantly reduced (P<0.01; Fig. 6-E), whereas the expression of Bax, PARP-1, cleaved caspase-3 and MMP-13 was significantly increased in the IL-1β group compared with the control (P<0.01; Fig. 6C-E). When chondrocytes were transfected with pLKO.1-cTHRc1-shRNA or SP600125 was added prior to IL-1β, Bcl-2 expression was significantly higher compared with the IL-1β group (P<0.01; Fig. 6E); however, MMP-13, Bax, PARP-1 and cleaved caspase-3 expression were significantly lower (P<0.01; Fig. 6C-E). Furthermore,
pLKO.1-CTHR1-shRNA or SP600125 treatment also significantly inhibited IL-1β-induced caspase-3 activation (P<0.01; Fig. 6F), suggesting that it may have a protective effect on chondrocyte apoptosis.

Discussion

OA is a degenerative joint disorder with multifactorial risk factors, including genetic and epigenetic factors, age, sex, ethnicity and obesity (7,19). CTHR1 protein is expressed in a number of embryonic and neonatal tissues, including developing cartilage and bone (20). In a previous study, CTHR1 was reported to stimulate bone formation in vitro and it was suggested that the endogenous expression of CTHR1 contributes to effective osteogenic differentiation by affecting cell proliferation and increasing the expression of osteogenic marker genes (21). CTHR1 is associated with the severity of murine collagen antibody-induced arthritis (22) and inhibition of osteoclast differentiation (23). CTHR1 is upregulated in patients with OA (15), suggesting a correlation between CTHR1 and arthritis progression. The results of the present study demonstrate that CTHR1 is more highly expressed in the joint fluid of patients with OA compared with normal joint fluid samples. However, the molecular mechanisms of and the role of CTHR1 in human chondrocytes and OA progression remain to be elucidated.

IL-1β has been implicated in chondrocyte apoptosis and the degeneration of articular cartilage (24) and is of great importance for the mechanisms of degeneration and degradation of articular cartilage in OA (25); as such, IL-1β was used in the present study to determine the function of CTHR1 in OA. It has previously been reported that IL-1β levels are increased in the synovial fluid of patients with OA and this is associated with chondrocyte apoptosis, resulting in cartilage destruction and pain (26,27), which is consistent with the findings of the present study. Karaliotas et al (28) reported that the ratio of Bax/Bcl-2 was increased in patients with OA compared with a normal cartilage control group, suggesting apoptosis induction. Furthermore, caspase-3 and MMP-13, which is commonly used as a marker of chondrocyte apoptosis and matrix degradation (29), were downregulated when CTHR1 levels were reduced. This indicates the potential role of CTHR1 downregulation in IL-1β-induced apoptosis inhibition, suggesting that the suppression of chondrocyte apoptosis may, in part, result in increased chondrocyte proliferation.

To investigate the underlying mechanisms of CTHR1 downregulation, its effect on JNK1/2 activation in chondrocytes was assessed. A previous study also provided evidence that the JNK1/2 signaling pathway is of great importance for regulating cell apoptotic signals in a number of cells (30) and is activated following IL-1β stimulation in osteoarthritic cartilage but not in normal cartilage (31). These studies serve to increase our understanding of the molecular mechanisms of chondrocyte proliferation, differentiation and apoptosis, and are in agreement with the in vitro experiments performed in the present study. Furthermore, JNK1/2 inhibitor SP600125 treatment and CTHR1 downregulation were demonstrated to inhibit IL-1β-induced JNK1/2 activation, whereas CTHR1 upregulation mimics the effect of IL-1β on chondrocyte apoptosis and JNK1/2 activation, suggesting that JNK1/2 signaling is associated with CTHR1-mediated chondrocyte proliferation and apoptosis.

The results of the present study suggest that CTHR1 downregulation promotes chondrocyte proliferation and inhibits apoptosis by directly regulating the JNK1/2 signaling pathway in IL-1β-induced primary rat chondrocytes. CTHR1 upregulation may strengthen disease progression in an in vitro rat model of OA. CTHR1 may serve as a novel therapeutic target for the regeneration of cartilage in patients with OA.

Competing interests

The authors declare that they have no competing interests.

References


