Knockdown of DAPK1 attenuates IL-1β-induced extracellular matrix degradation and inflammatory response in osteoarthritis chondrocytes via regulating the p38 MAPK-signaling pathway

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Objective: To reveal the possible effects of death-associated protein kinase 1 (DAPK1) on the progression of osteoarthritis (OA) and the potential underlying mechanism.

Methods: The expression of DAPK1 in OA and normal samples and interleukin (IL)-1β-stimulated chondrocytes was analyzed by quantitative real-time polymerase chain reaction and Immunoblot assay. Cell viability, proliferation, and apoptosis in DAPK1-knockdown cells stimulated with IL-1β were detected by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution, 5-ethynyl-2′-deoxyuridine staining and flow cytometry. The chondrocyte degradation and inflammatory response in IL-1β-induced chondrocytes were investigated by Immunoblot analysis and enzyme-linked-immunosorbent serologic assay. In addition, the effect of DAPK1 on p38 mitogen-activated protein kinase (MAPK) activation was analyzed by immunoblot assay.

Results: This study revealed that DAPK1 was highly expressed in OA patients and IL-1β-stimulated cartilage. Down-regulation of DAPK1 enhanced IL-1β-induced chondrocyte proliferation. DAPK1 knockdown inhibited IL-1β-induced chondrocyte degradation. In addition, DAPK1 depletion inhibited IL-1β-induced chondrocyte inflammation. Mechanically, it was revealed that down-regulation of DAPK1 could inhibit the p38 MAPK pathway, and therefore affected progression of OA.

Conclusion: DAPK1 knockdown attenuates IL-1β-induced extracellular matrix degradation and inflammatory response in OA chondrocytes by regulating the p38 MAPK pathway.

KEYWORDS
death-associated protein kinase 1 (DAPK1); IL-1β; inflammation; osteoarthritis (OA); P38 MAPK pathway
Introduction

Osteoarthritis (OA) is a chronic degradative disease of the joints characterized by degeneration of the articular cartilage. It affects approximately 40% of people over the age of 70 years and is associated with an increased risk of complications and death. OA is caused by dysregulation of the cartilage homeostasis because of a variety of physiological and mechanical factors. A hallmark of OA is degradation of the extracellular matrix (ECM). Apoptosis and inflammatory response of chondrocytes also greatly promote degradation of ECM. Therefore, effective inhibition of pathways may be a promising approach for maintaining cartilage integrity.

Death-associated protein kinase 1 (DAPK1) is a calmodulin-regulated serine/threonine kinase with powerful biological functions. Studies have shown that pharmacological inactivation or gene ablation of DAPK1 makes renal tubular cells resistant to lipopolysaccharide (LPS)-induced inflammation in the presence of hypoxia, and targeting DAPK1 effectively protects mice from sepsis-induced acute kidney injury. DAPK1 knockdown reduced interleukin (IL)-1β maturation and caspase-1 activation in microglia and improved memory impairment in mice injected with Aβ25-35. Knockout or inhibition of DAPK1 had a protective effect on lung injury.

In addition, RNA-seq data analysis showed that DAPK1 was highly expressed in chondrocytes of OA patients. DAPK1 binds to p38 mitogen-activated protein kinase (MAPK) and selectively promotes p38 MAPK activation, thereby enhancing MAPK-activated protein kinase 2 (MK2) phosphorylation. The inhibition of p38 MAPK pathway inhibits inflammatory response in OA. In addition, the increased expression of matrix metalloproteinase (MMP) depends on the activity of p38 MAPK in the context of cartilage matrix degradation. However, the possible effects of DAPK1 on the progression of OA are still unclear.

This study aimed to reveal the possible effects and potential mechanisms of DAPK1 on the progression of OA. We found depletion of DAPK1 attenuates IL-1β-induced ECM degradation and inflammatory response in OA chondrocytes by regulating the p38 MAPK-signaling pathway.

Materials and Methods

Isolation and culture of cells

The protocols used in this study were approved by the Ethics Committee of Changzhi Medical College. Human chondrocyte samples were isolated from the knee articular cartilage of OA patients. Chondrocytes were collected and maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. OA were stimulated with 0 ng/mL, 5 ng/mL, 10 ng/mL, and 20 ng/mL quercetin. Human DAPK1 short hairpin (sh)RNA or control shRNA was constructed by using the GeneSuppressor System (Imgenex, CO, USA). Cells were transfected using lipofectamine 2000 according to the manufacturer’s instructions.

Cell viability

Cells were plated at a density of 3×10^4 cells/well into 96-well plates. Cell viability was assessed with the addition of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. Cells were incubated for another 4 h and the insoluble pellets were dissolved with dimethyl sulfoxide (DMSO).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cellular total RNA was extracted with TRIzol reagents (Thermo, Rockford, USA). Total RNA was reverse-transcribed into complementary DNA (cDNA) using Moloney Murine leukemia virus (M-MLV) reverse transcriptase (RTase) (Promega Corporation, Madison, WI, USA). The relative expressions of target genes were detected by qRT-PCR by using the following primers: human DAPK1: CCAGACTGTCCTCCACC, TCTTCACACTCAGTTC; IL-6: TCCTACCCCAACTTCCAATGCTC, TTGATGCTTCTTGGCTCTGACC; tumor necrosis factor (TNF)-α: AAAGGA CACCATGAGCAGGAAAG, GCAGCAGCGCAGGAAAG; cyclooxygenase-2 (COX-2): GATGACGAGCGACTGTTCCA, TGGTACCCGCTGAGGTGTTG; inducible nitric oxide synthase (iNOS): ACCACCCCTCTTGGTCAAC, CAATCCCACAAGCTGCT CAA; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): TCAAGGCTGAAACGGGAAG, TGACTCCAGGAG GACTCA. Mice Dapk1: AACACCTCCCTCTACCCAGCTG, ACCACATGTCCTCCTGTTG. Mice GAPDH: GGTACCAGGGCT GTCTTCTTCTG, AGCCTTGACTGTGCGGTGAAC.

Immunoblot assay

Proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). Then the samples were collected and electrophoresed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto membranes, and blocked by skimmed milk. Subsequently, membranes were incubated with primary antibodies targeting DAPK1 (mouse, 1:1000; Abcam, Cambridge, UK), MMP-3 (mouse, 1:1000; Abcam), MMP-9 (mouse, 1:1000; Abcam), MMP-13 (mouse, 1:1000; Abcam), collagen II (mouse, 1:1000; Abcam), p-p65 (mouse, 1:1000; Abcam), p-p65 (mouse, 1:1000; Abcam), p38 (rabbit, 1:1000; Abcam), p38 (rabbit, 1:1000; Abcam), phosphorylated (p)-MK2 (rabbit, 1:1000; Abcam), MK2 (rabbit, 1:1000; Abcam), and β-actin (mouse, 1:10000; Abcam) for 1 h. Ultimately, the membranes were conjugated with anti-mouse immunoglobulin G (IgG) and anti-rabbit IgG (Abcam) for 1 h. Specific proteins were visualized with enhanced chemiluminescence detection kit (ECL; Thermo, Rockford, IL, USA).

5-Ethynyl-2’-deoxyuridine (EdU) staining

The proliferation of OA chondrocytes was evaluated by EdU staining kit. OA chondrocytes were plated into 96-well, followed by indicated treatment. Then cells were added with 100-μL 50-μM EdU solution for 2 h. Cells were then fixed...
with 4% paraformaldehyde and incubated with 2 mg/mL glycine solution for 5 min. Cells were permeabilized with 0.5% phosphate-buffered saline solution with 0.05-0.1% Tween™ 20 (PBST).

Flow cytometry (FCM) analysis

Chondrocytes were washed twice in cold PBS, followed by resuspension in 100-μL Annexin V kit. FITC Annexin V and propidium iodide (PI), 5 μL, were then added, and the cells were incubated for 15 min.

Enzyme-linked-immunosorbent serologic assay (ELISA)

The concentrations of TNF-α, PEG2, IL-6, and nitric oxide (NO) in cell lysates were measured with ELISA following the manufacturer’s protocol.

Statistical analysis

GraphPad 6.0 was used for statistical analysis. Three replicates were performed for each experiment. One-way ANOVA and Student’s t-test were used for statistical comparisons. P < 0.05 was taken as statistically significant. Data were presented as mean ± standard error of mean (SEM).

Results

DAPK1 was up-regulated in IL-1β-induced chondrocytes

We first evaluated the expression of DAPK1 in OA and OA cells. The results showed that DAPK1 was highly expressed in the cells of OA patients (Figures 1A and B). Cell viability of chondrocytes in response to increasing level of IL-1β was detected by MTT assay. The results indicated that high level of IL-1β resulted in reduced cell viability in chondrocytes (Figure 1C). Furthermore, DAPK1 cells were found enriched in IL-1β-induced chondrocytes (Figures 1D and E). Collectively, DAPK1 was up-regulated in OA induced by IL-1β.

DAPK1 knockdown promotes cell viability in IL-1β-induced chondrocytes

In order to evaluate the role of DAPK1 in chondrocytes induced by IL-1β, DAPK1 was knocked down in cells with short hairpin (sh)RNA transfection. As expected, IL-1β-induced DAPK1 level and shRNA transfection further reduced the level of DAPK1 (Figure 2A). The role of DAPK1 in cell viability exposed to IL-1β was detected by MTT assay. DAPK1 knockdown enhanced cell viability in chondrocytes in response to IL-1β (Figure 2B). DAPK1 knockdown also promoted the cell proliferation of chondrocytes in response to IL-1β (Figure 2C). Furthermore, DAPK1 knockdown also protected chondrocytes from apoptosis induced by IL-1β (Figure 2D). Collectively, DAPK1 knockdown promoted cell viability in IL-1β-induced chondrocytes.

DAPK1 knockdown reverses cartilage matrix degradation in IL-1β-induced chondrocytes

The expressions of MMP-3, MMP-9, MMP-13, and collagen type II in response to IL-1β stimulation and DAPK1 knockdown were detected by Immunoblot assay. Our results showed that IL-1β treatment up-regulated MMP-3, MMP-9, MMP-13, and collagen II caused at protein levels (Figure 3). In addition, DAPK1 knockdown reversed these alterations induced by IL-1β (Figure 3). Taken together, these results indicated that DAPK1 knockdown could effectively reverse cartilage matrix degradation.

Figure 1 DAPK1 was up-regulated in IL-1β-induced chondrocytes. (A) and (B) The mRNA and protein level of DAPK1 in OA and normal samples. (C) Cell viability exposed to increasing level of IL-1β. (D) and (E) The mRNA and protein level of DAPK1 in response to increasing level of IL-1β. *P < 0.05, **P < 0.01, and ***P < 0.001.
Figure 2 DAPK1 knockdown promotes cell viability in IL-1β-induced chondrocytes. (A) The protein levels of DAPK1 in control, IL-1β, IL-1β + shNC, and IL-1β + shDAPK1 cells. (B), (C), and (D) Cell viability, proliferation, and apoptosis in control, IL-1β, IL-1β + shNC, and IL-1β + shDAPK1 cells. *P < 0.05, **P < 0.01, and ***P < 0.001.

DAPK1 knockdown improves IL-1β-induced cell inflammation

In order to explore inflammatory response in IL-1β-induced chondrocytes, levels of IL-6, PEG₂, NO, and TNF-α were examined in cells. IL-1β significantly induced the levels of IL-6, PEG₂, NO, and TNF-α (Figure 3A). It was further discovered that DAPK1 knockdown relieved cellular inflammation as identified by the reduction of these cytokines (Figure 3A). The mRNA level of IL-6, PEG₂, NO, and TNF-α after DAPK1 knockdown showed the same phenomenon (Figure 3B). Therefore, we speculated that DAPK1 knockdown was responsible for inhibited inflammation in LPS-treated cells.

Down-regulation of DAPK1 could inhibit the p38 MAPK pathway

Since DAPK1 mediates cell inflammation, the involvement of DAPK1 in p38 MAPK pathway was evaluated. IL-1β increased the levels of p-p38, p-MK2, and p-p65. The IL-1β-induced alterations were abrogated by DAPK1 knockdown (Figure 5A). We further revealed the decreased phosphorylation levels of nuclear factor kappa B (NF-κB) after DAPK1 knockdown (Figure 5B). Therefore, down-regulation of DAPK1 could inhibit the p38 MAPK pathway.
DAPK1 affects OA progression

Chondrocytes were obtained by trypsin and collagenase digestion. Through qRT-PCR and Immunoblot assay, it was revealed that DAPK1 was highly expressed in OA patients and IL-1β-induced chondrocytes. By performing Immunoblot, MTT, and FCM assays and Edu staining, it was revealed that DAPK1 knockdown enhanced IL-1β-induced chondrocyte proliferation. Further, through Immunoblot assay, ELISA, and qRT-PCR analysis, our results confirmed that down-regulation of DAPK1 inhibited IL-1β-induced chondrocyte degradation and inflammatory response. Therefore, these results indicated that DAPK1 was a critical regulator in OA progression. The multiple biological functions of DAPK1 have been widely revealed.

Discussion

Osteoarthritis is a common disease of the joints and is mainly caused by cartilage damage. It is the main cause of disability in middle-aged and elderly people, with increasing incidences and lack of effective treatment. The etiology of OA is still unclear. In order to further improve therapeutic outcomes in patients, it is necessary to have a deeper understanding of the pathogenesis of OA and discover more key proteins. In this study, we revealed that DAPK1 knockdown attenuated IL-1β-induced ECM degradation and inflammatory response in OA chondrocytes.

In this study, cartilage tissue was obtained from the knee joint of 1-week-old Sprague Dawley rats, and chondrocytes were obtained by trypsin and collagenase digestion. Through qRT-PCR and Immunoblot assay, it was revealed that DAPK1 was highly expressed in OA patients and IL-1β-induced chondrocytes. By performing Immunoblot, MTT, and FCM assays and Edu staining, it was revealed that DAPK1 knockdown enhanced IL-1β-induced chondrocyte proliferation. Further, through Immunoblot assay, ELISA, and qRT-PCR analysis, our results confirmed that down-regulation of DAPK1 inhibited IL-1β-induced chondrocyte degradation and inflammatory response. Therefore, these results indicated that DAPK1 was a critical regulator in OA progression. The multiple biological functions of DAPK1 have been widely revealed.
its nuclear translocation and further promoting bone marrow adipogenesis. Similarly, we here revealed that DAPK1 could also mediate p38 MAPK pathway.

In addition, the knockdown or inhibition of DAPK1 had a protective effect on lung injury, reduced lung injury score, resolved lung inflammation, and inhibited the percentage of apoptosis of alveolar epithelial cells. It was also revealed that ablation of DAPK1 inhibited IL-1β-induced chondrocyte inflammatory response. Another study indicated that pharmacological inactivation or gene ablation of DAPK1 made renal tubular cells resistant to LPS-induced inflammation. We should next use the inhibitor of DAPK1 and detect its effects on the progression of OA.

It was also revealed that DAPK1 mediated p38 MAPK pathway and affected OA progression. DAPK could bind to p38 MAPK and selectively promoted p38 MAPK activation, thereby enhancing MK2 phosphorylation. Inhibition of p38 MAPK pathway could inhibit inflammatory response in OA. Therefore, DAPK1 might affect inflammation in OA by this pathway. Here, we also revealed that down-regulation of DAPK1 attenuated IL-1β-induced ECM degradation in OA chondrocytes. These results suggested that p38 MAPK was a fundamental target in OA.

Conclusion

The present study revealed that down-regulation of DAPK1 improved IL-1β-induced chondrocyte degradation, inflammatory response, and apoptosis, and promoted chondrocyte proliferation, thereby alleviating OA by regulating p38 MAPK pathway.

Competing Interests

The authors stated that there were no conflicts of interest to disclose.

Statement of Informed Consent

Written informed consent was obtained from legally authorized representative(s) for anonymized patient information to be published in this article.

Data Availability

The authors declare that all data supporting the findings of this study are available in the paper, and any raw data can be obtained from the corresponding author upon request.

Author Contributions

Jie Wei and Chen Gao designed and carried out the study. Jie Wei, Chen Gao, Ke Hu, Mingyue Li, Jingshi Li, Mengman Shen, and Shuyu Zhang supervised collection, analysis, and interpretation of data. Jie Wei and Chen Gao prepared and reviewed the draft of the manuscript for publication. All authors read and approved the final manuscript.

References


