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CDKN1A down-regulation of inflammasomes and pro-inflammatory cytokines in pyroptosis of cartilage cells

M Sharma, H Mishra, S Singh, K Longkumer, M Dhuria[1*]

Abstract

Osteoarthritis is the most common chronic degenerative disease worldwide; it mainly affects elderly people. This disease can involve nearly any joint in the human body, and the most common symptoms include joint pain and disordered articular functions. Inflammasomes (NLRP3) which are induced by nuclear factor kappa B (NF-κB) signaling and can convert interleukin-1β (IL-1β) and IL-18 into mature proinflammatory cytokines are considered a factor in low-grade inflammatory pathology. This study aimed to explore the mechanisms underlying CDKN1A-in osteoarthritis. Chondrocytes were collected and isolated from 22 patients with osteoarthritis (average age 50.22 ± 2.15) and healthy volunteers (average age 51.12 ± 2.34) were enrolled as the control group from June 2021 to June 2023. mRNA expression levels of CDKN1A, NLRP3, and cleaved-Caspase1) were detected by real-time PCR. Cell activity was calculated with CCK-8. It has been found that CDKN1A regulates DNA damage repair, which contributes to the improvement of osteoarthritis by regulating the pyroptosis of cartilage cells. However, the exact mechanistic effects are still unknown.

Keywords: Osteoarthritis; CDKN1A; Cohort Study; Inflammasomes

*Corresponding author email: Dhuria.md@manipal.edu.
[1] Department of Immunology, Melaka Manipal Medical College, India
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Introduction

Osteoarthritis (OA) is the most common chronic degenerative disease worldwide. Although OA is highly prevalent [1], it mainly affects elderly people. This disease can involve nearly any joint in the human body, and the most common symptoms include joint pain and disordered articular functions [2]. The pathological changes associated with OA affect all tissues in the joint and include cartilage degeneration, subchondral sclerosis, variable degrees of synovial inflammation, osteophyte formation, and hypertrophy of the whole joint capsule [3]. Inflammasomes such as NLRP3 (NLR family, pyrin domain containing 3; NLR refers to “nucleotide-binding domain, leucine-rich repeat”), which are induced by nuclear factor kappa B (NF-κB) signaling and can convert interleukin-1β (IL-1β) and IL-18 into mature proinflammatory cytokines, are considered a factor in low-grade inflammatory pathology [4]. Among these

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inflammasomes, which comprise NLRs (NLRP1, NLRP3, and NLRC4), absent in melanoma 2 (AIM2), IFN-inducible protein 16 (IFI16) and pyrin, the NLRP3 inflammasome is the best characterized [5]. This complex comprises NLRP3, apoptosis-related speck-like protein containing (ASC) a caspase recruitment domain (CARD) and procaspase-1 [6], suggesting that pyroptosis may be closely involved in the pathological changes associated with OA. Furthermore, the frequency of OA pain was indicated to be mediated by aberrantly overexpressed cytokines and to be associated strongly with inflammatory severity, especially in soft tissues such as the synovium [7], which could lead to hypersensitivity with exaggerated pain (hyperalgesia) in response to noxious stimuli or innocuous stimuli that are perceived as painful (allodynia) [8]. Several studies have investigated the roles of pyroptosis in different diseases, particularly cardiovascular diseases[9]. Existing evidence has revealed significant increases in caspase-1 expression in vulnerable plaques and ruptured lesions associated with acute coronary events in atherosclerosis [10]. Additionally, monocyte/ macrophage pyroptosis may also induce an amplified inflammatory response and promote plaque rupture and thrombosis, which can induce acute cardiovascular events [11]. Moreover, vascular smooth muscle cell pyroptosis may suppress the healing of vascular injuries and reduce the stability of atherosclerotic plaques [12]. Cardiomyocyte and cardiac fibroblast pyroptosis is also involved in diabetic cardiomyopathy [13], cardiac hypertrophy [14] and ischemic heart disease. Existing evidence suggests that inflammasomes such as NLRP3 can be activated by fatty acids and high glucose levels, leading to chronic intestinal inflammation [15] and colorectal cancer. These findings indicate that pyroptosis plays a significant role in digestive tract inflammation as well as in obesity-associated carcinogenesis. Pyroptosis, a highly inflammatory mode of cell death, can also trigger autoimmune diseases such as systemic lupus erythematosus (SLE) [2]. LPS from gram-negative bacterial infection induces pyroptosis by activating caspase-4/5/11, contributing to infectious diseases such as sepsis [7]. Pyroptosis also plays a role in inflammatory joint diseases such as rheumatoid arthritis (RA) [17] and OA [5]. This indicates that pyroptosis may contribute to OA pain by releasing related cytokines [18]. Pyroptosis is a proinflammatory cell death triggered by inflammatory corpuscles, which will induce cell rupture and release cell contents, similar to necrosis, associated with the development, homeostasis, and senescence of cells [19]. Therefore, it is of great significance to determine the role of pyroptosis of cartilage cells in osteoarthritis and to explore its regulatory mechanism for the treatment of osteoarthritis [11]. Transcription factor DKN1A is an important regulator involved in a variety of physiological and pathological processes. There has been no clear study on the pyroptosis of cells, especially cartilage cells.

This study aimed to explore the mechanisms underlying CDKN1A in osteoarthritis.

Patients and Methods

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TD-SC Isolation Culture The donor was a 12-year-old girl patient who was diagnosed with knee trauma. The patient provided her or her guardians with informed consent. TD-SCs from traumatic knees were obtained by outgrowth. At 10 days, the initially attached cells were cultured in complete growth medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin antibiotics, and 1% L-glutamine. The medium was changed every 3-4 days. Upon ~80% confluence, the chondrocytes were replated, and culture expanded until P3.

Gene Expression Analysis

Total RNA was isolated from cells and cartilage tissues using Trizol Reagent and reverse transcribed into cDNA with a reverse transcription kit. cDNA was amplified using a SYBR Green PCR Mix on a 7900HT Fast Real-Time PCR system. Gene expression levels were measured by relative quantification with glyceraldehyde-3-phosphate dehydrogenase as a reference. The following cycle times were used: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and 30 s at 72 °C.

Gene Expression Analysis

Pyroptosis and inflammation tests were performed multiple times to estimate the significance of the difference between the internal control group and the treatment group result. Gene expression patterns in cells transfected with the sgRNA-CNTL vector and cells separately transfected with sgRNA-GSDMC were analyzed. Total RNA from cells was isolated. cDNA was synthesized using the PrimeScript™ RT reagent kit. Real-time quantitative PCR was performed utilizing TB Green® premix.

In real-time quantitative PCR, genes associated with pyroptosis (GSDMD, Gasdermin E unique region, and N-terminal fragment), inflammation (NLRP3, IL-1β, and TNF-α), and CDKN1A, the target gene of p21, were detected with forward and reverse primers. The relative gene expression levels were calculated using the Livak (2-ΔΔCt) equation. GSDMD and GSDMD-N were significantly expressed after sgRNA-GSDMC transfection, but there were no significant differences in expression. The results showed that the internal control expression of the pyroptosis-related protein NLRP3, IL-1β, and TNF-α genes was significantly increased in the paroxysmal joint pre-cultured for 24 h compared with the control group; GSDMD gene expression was not significantly different ($P > 0.05$). The results showed that mRNA and protein expression CDKN1A and p21 were significantly decreased in the GSDMC intervention group compared with the normal control group, which is consistent with the results of Western blot analysis. Western blot analysis found that the expression of the CDKN1A and p21 genes was lower in chondrocytes transfected with siGSDMC than in the normal control.

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Cell Culture Techniques

Cell Culture Techniques Some of the procedures have been certified for use with in vitro cultured human cell lines. It should be noted that the initial cell sources used may affect the results, even though the expected levels of protein expression or activity may seem consistent. There are many variations, including the nature of the cells, culture methods, and cell sub-lines, which can be found within the scientific community. Therefore, the information provided here is a general summary. Nowadays, researchers are beginning to focus on local DNA and protein expression. In vitro studies help us evaluate signaling pathways involved in cellular processes and potential treatments for cartilage catabolic disorders. There are several methods available for chondrocyte decontamination using various enzymes. The procedures are as follows: 1. Remove the cartilage mass from the body. 2. Tear the cartilage into smaller pieces using a scalpel. 3. Place the pieces in a clean petri dish with PBS and remove any blood using tweezers. 4. Wash the chondrocyte-containing pieces with PBS. 5. Next, place the chondrocytes in 1 mg/helper PBS of tired collagenase at 37°C in a shaking incubator (with 5% carbon dioxide) and shake continuously for one hour to allow the enzymes to break down the collagen. 6. After one hour, wash the cells with PBS and wait for twenty minutes to allow the chondrocytes to settle at the bottom of the dish. 7. Pipette the chondrocytes into a 50 ml tube. 8. Discard the top half of the cell suspension by gently aspirating the liquid in the petri dish.

Statistical Analysis

The statistical comparison was carried out by one-way ANOVA between the two groups, and $P<0.05$ means that the difference was statistically significant. Data analysis was performed with GraphPad Prism 7.0 soft-ware.

Results

In this study, we report that CDKN1A does not inhibit caspase-1 protein but suppresses the expression of NLRP3 and its upstream ASC apoptosis-associated speck-like protein containing CARD at the mRNA level. Hence, CDKN1A silencing by miR-140 is essential for the excessive formation of NLRP3 inflammasome and caspase-1 impairment by inflammation and pyroptosis. Pyroptosis is a caspase-1 and caspase-11/4-mediated cell death which occurs in response to cytosolic lipopolysaccharide and inflammasome-activating damage signals. Inflammasomes sensing cellular dye and variable perturbations are highly multicomponent cytosolic protein complexes. The activation of NLRP3 inflammasome depends on apoptosis-associated speck-like protein containing CARD (ASC) adapter, NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) sensor and the apoptosis-NF-κB pathway inhibitory kinase program need particular contact-dependent changes between NLRP3 sensors. Active NLRP3 inflammasomes oligomerize, recruit procaspase-1 zymogens and trigger inflammatory Casp1 and gasdermin-D cleavage.

Apoptosis-associated speck-like protein containing CARD (ASC) sensors also recruit pro-caspase protease 8. Active caspase-1 zymogens (not inactive) are cysteine proteases that cleave and activate secreted toxic gasdermin-D protein to form perforation pores in dying cells and the surrounding non-vacuolar membranes. The CDKN1A (cyclin-dependent kinase inhibitor 1A, also known as p21) is a universal inhibitor of cyclin-dependent protein kinases that inhibit the cell cycle and is induced by a CDKN1A-regulated inhibitor responses DNA damage transduction signaling protein Chk2 (CDC25 family CMD conduit regulatory protein) (CDKs). CDKN1A acts as a gating mechanism to control pyrin-inflammasome assembly and pyroptosis in the course of cellular accumulation of the oncogenic p21 (CDKN1A is an adenocarcinoma hormone receptor that is highly active in inflammatory cytokines interleukin 1β (IL-1β)).

Discussion

OA is the most common degenerative joint disorder and can affect nearly any small or large joint in the body [21]. OA is now considered to have complex etiology, and these multiple factors can lead to similar outcomes of joint destruction and clinical features [22]. In a study of OA patients older than 50 years of age in North America and Europe, approximately 60%, 33% and 5% reported involvement of joints of the hand, knee and hip, respectively [23]. An age older than 40 years is associated with a greater likelihood of developing OA. Moreover, OA is more frequent in women than in men at any age older than 50 years (i.e., postmenopausal women), and this sex difference mainly involves the hand (e.g., distal interphalangeal) and knee joints [11]. The risk factors for OA include factors related to individual susceptibility, as well as those affecting biomechanical joint stability [2]. Obesity may also be a risk factor for OA, as it increases stress on weight-bearing joints such as the hips and knees. Additionally, fat cells produce potentially harmful inflammatory proteins that may target the joints. The genetics underlying OA are complex but highly significant and may be related to changes in important molecular pathways [24]. The genes encoding inflammatory and catabolism-related proteins are upregulated during OA, primarily through signal transduction involving nuclear factor-κB (NF-κB), mitogen-activated protein kinase, and other inflammation and stress-induced pathways [25]. Joint injuries, such as those involving the ligaments and meniscus, can increase the fragility of the joint and increase the risk of osteoarthritis. Even injuries that occurred many years ago can increase the risk of OA. Moreover, joint malformation or cartilage defects can increase the risk of OA [26]. The symptoms of OA include pain, transient morning stiffness, and a grating sensation when walking. End-stage OA is characterized by instability and physical disability, which have tremendous negative effects on the quality of life [7]. The lack of efficient and curative treatment options for OA and the ever-evolving pathophysiological and risk factors present significant challenges. Because its exact pathogenesis has not been fully revealed, OA is characterized as a whole-joint disease associated with pathological changes in all involved tissues. The pathology of OA includes the progressive loss and destruction of cartilage,

sclerosis of the subchondral bone, formation of osteophytes, variable degrees of synovial inflammation, degeneration of the ligaments and meniscus, and hypertrophy of the whole joint capsule[3]. Initially, the risk factors that cause joint instability, such as ligament injury and excessive body weight, induce the excessive secretion of transforming growth factor β1 (TGF-β1) in the subchondral bone. An excess of this cytokine leads to the uncoupling of bone formation and resorption, which is accompanied by angiogenesis, sensory innervation [6], bone cavity formation and sclerosis [28]. Articular cartilage degeneration is the primary concern in OA. Normally, in an environment with low oxygen, chondrocytes exhibit a low-grade turnover rate to maintain an anabolic-catabolic balance in the cartilage [27]. In OA, however, the expression of genes that encode proteins associated with inflammatory and catabolic responses is upregulated primarily via signal transduction pathways involving NF-κB, mitogen-activated protein kinase and other factors that are activated by inflammation [28]. This transcriptional activity increases the production of primary inflammatory cytokines, such as IL-1β and tumor necrosis factor α (TNF-α), which play critical roles in OA pathology. IL-1β and TNF-α stimulate chondrocytes to release cartilage-degrading enzymes such as metalloproteinases 1, 3 and 13 (MMP1, MMP3 and MMP13) and a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4) and ADAMTS5 [31]. These enzymes digest aggrecans and type II collagen to degrade the cartilage matrix. IL-1β and TNF-α also contribute to inflammatory chondrocyte death [2]. Together, these processes lead to a gradual loss of cartilage and the formation of osteochondral fissures, which allow the formation of new endochondral bone accompanied by angiogenesis and sensory innervation [8]. These processes may be closely associated with OA pain. During the late stage of OA, patients lose joint function and present with an immensely narrowed joint space containing little remaining cartilage, especially in the knee and hip joints.

To date, a limited amount of literature has described the role of the NLRP3 inflammasome in OA pathogenesis. Since strong evidence shows that NLRP3 is a potential marker in other diseases such as RA [9], atherosclerosis [11], gout and colorectal cancer, we detailed the evidence that indicates the association of NLRP3 with OA in the following sections. Cartilage degeneration is the most well-known pathological change associated with OA. The joint tissues of patients harboring OA risk factors, such as metabolic disorders, aging, infectious joint diseases, and injuries, can generate several kinds of DAMPs or PAMPs, including adipokines, microcrystals, and uric acid. Previous studies have shown that, both BRAC1a and BRAC1b activate CDKN1A transcriptionally in both p53-dependent and non-dependent ways, and can also regulate CDKN1A expression through phosphorylation of SMADs. Moreover, the activation of mitogen-activated protein kinase (MAPK) kinase pathway contributes to the regulation of TGF-β by CDKN1A [29].

Furthermore, in cancer cells, High levels of c-myc protein inhibit the binding of TGF-β to SMAD, replacing the activator from the p21promoter and thus activating CDKN1A. Ligase ubiquitination and proteasomal degradation are two mechanisms that negatively regulate p53

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and CDKN1A. MKRN1 is an E3 ubiquitin ligase, which increases the degradation of p53 through ubiquitination and reduces the transcription of p53on CDKN1A [30]. In addition, MKRN1 can directly bind to CDKN1A and degrade CDKN1A in p53-independent manner. A study has reported CDKN1A regulation on programmed cell death, namely, apoptosis. CDKN1A can regulate apoptosis in many types of cells. For ex-ample, it has been reported that CDKN1A overexpression in breast cancer cell lines reduces cell sensitivity to infrared (IR)-induced apoptosis. Further experimental studies show that p21can protect cells from IR-induced apoptosis by inhibiting CDKs [31]. Additionally CDKN1A plays an important role in cell cycle progression, expression of DNA re-pair and apoptosis-regulated genes, including E2F families, NF-κB, c-myc, STAT, and p300/CPB. A number of studies have shown that, p21plays a key role in tumorigenesis and promotion. The inhibition of apoptosis is the most famous oncogenic function of CDKN1A. Other studies showed that study, CDKN1A enhanced radio resistance in lung adenocarcinoma cells. CDKN1A upregulation following radiotherapy promoted lung adenocarcinoma cell survival, as evidenced by the increased viability of irradiated CDKN1A-overexpressing lung adenocarcinoma cells [32]. Furthermore, following CDKN1A overexpression, the irradiated mice's serum caspase-1 activity and IL-1β levels dropped. Cellularly, there was a decrease in the number of pyroptotic cells, along with a drop in caspase-1 activity, LDH release, IL-18 and IL-1β levels, and ASC speck count. Following CDKN1A overexpression, the NLRP3 and AIM2 inflammasomes' activation in the irradiated A549 cells diminished [33].

Conclusions

Collectively, the findings present the initial data to highlight that cartilage cells expressing osteoarthritic markers might undergo pyroptosis because of CDKN1A deposition over prolonged periods due to ageing. CDKN1A down-regulation not only inhibits NLRP1/NLRP3 inflammasomes up-regulation but also abrogates activation of the apoptotic caspase-1 in mitochondria and nucleus. Consequently, CDKN1A down-regulation could have potential as a treatment strategy for osteoarthritis. Overexpression of miR138 could attenuate CDKN1A as a miRNA therapeutic process. Future studies need to further explore the roles of CDKN1A in OA using simple in vitro cell culture models, including knockdown of CDKN1A or up-regulation of miR138. We believe that high throughput screening as well as in silico analysis of target genes/pathways would further our understanding of the roles of CDKN1A and have potential implications not only in therapies, but also in the diagnosis and prognosis of OA in future.

Conflict of Interest

No conflicts of interest were declared by the authors.

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Ethics Statement

Not applicable.

Authors' contributions

All authors shared in the conception and design and interpretation of data, drafting of the manuscript and critical revision of the case study for intellectual content and final approval of the version to be published. All authors read and approved the final manuscript.

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