Down-Regulation of MCT1 Ameliorates LPS-Induced Cell Injury in Murine Chondrocyte-like ATDC5 Cells by Regulation of PFKFB3

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Abstract: MCT1 is an important regulator in glycolysis and has significant effects on inflammatory responses and osteoclast differentiation etc. This study was to study the effects and mechanism of MCT1 in chondrocytes injury and inflammatory responses. ATDC5 cells with stably transfection of MCT1shRNA were treated with 5 μg/mL of LPS. Cell viability was determined by MTT assay. The mRNA and protein expressions were detected by qRT-PCR and western blotting, respectively. The concentrations of cytokines in culture medium were measured by ELISA. ROS generation was tested by 2,7-dichlorofluorescein diacetate (DCFH-DA). The results showed that MCT1 was increased by LPS treatment in ATDC5 cells in a dose dependent manner. MCT1 knockdown improved the survival of LPS-treated ATDC5 cells. MCT1 knockdown also decreased LPS-induced expression of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNFα) and oxidative stress mediators (iNOS, COX-2 and NOX-4) in ATDC5 cell. Importantly, PFKFB3 overexpression reversed the anti-inflammatory and anti-oxidative stress effects of MCT1 knockdown in LPS-induced ATDC5 cells. These results indicated that MCT1 knockdown increased the expression of inflammatory mediators and oxidative stress mediators induced by LPS through regulating PFKFB3. The study provides a potential target for the prevention or treatment of osteoarthritis (OA) and rheumatoid arthritis (RA).

Key words: Chondrocytes, MCT1, Osteoarthritis, PFKFB3

Introduction

Osteoarthritis (OA) is the most common chronic joint disease, which leads to pain and disability in the middle-aged and elderly people⁵. Cartilage degeneration is the main cause of OA, which may be induced by various factors including obesity, trauma and inflammatory diseases⁶. Therefore, it is urgent to explore the molecular mechanisms of OA, especially that of cartilage degeneration.

Chondrocytes are unique cells in articular cartilage that are responsible for the balance between the synthesis and degradation of extracellular matrix (ECM)⁷. In the process of cartilage degeneration, pro-inflammatory cytokines (such as TNFα, IL-1β, etc.) and oxidative stress mediators (such as COX-2, NO and ROS) may lead to the loss of major components of ECM and cartilage damage⁸.

6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), a regulator of human metabolism, plays an important role in inflammatory responses and is related to the occurrence of some diseases, such as diabetes and OA⁹. Studies have shown that PFKFB3 inhibition could reduce the expression of inflammatory factors such as IL-6, IL-8, and CXCL-10, thereby inhibiting the occurrence of synovial inflammation and joint destruction in rheumatoid arthritis⁹. Besides, researchers reported that PFKFB3 may sever as a switch that senses and controls redox homeostasis in cancer glycolysis, which confirmed that PFKFB3 could regulate oxidative stress homeostasis⁷.

Monocarboxylate transporters (MCTs) are important regulators in glycolysis by transporting lactate outside of the cellular membrane⁹. In recent years, the functional role of MCT1 in the tumor process, inflammatory response, oxidative stress and other aspects has attracted more attention⁹. It has been reported that MCT1 promotes the microglial classical activation and neuroinflammation by increasing PFKFB3 expression⁹. However, studies considering the role of MCT1 in OA were little reported. Based on these conclusions, we thus speculated that MCT1 could regulate inflammatory chondrocytes injury via PFKFB3. This study aims at spelling out the involvement of role PFKFB3 in the functional role of MCT1 in chondrocytes injury.

Materials and Methods

Cells culture and treatment

ATDC5 cells, isolated from mouse teratocarcinoma fibroblastic cells, were purchased from Nanjing Kebai Biotechnology Co., Ltd. (Nanjing, China) and cultured in DMEM/F12 (1:1) medium (Life Technologies, California, USA) supplemented with 5% FBS (Thermo Fisher Scientific, Inc., Massachusetts, USA) under 5% CO₂ at 37°C. ATDC5 cells were treated with various concentrations (2.5, 5 or 10 μg/ml) of LPS. ATDC5 cells with stably transfection of MCT1shRNA were treated with 5 μg/ml of LPS.
**Cell transfection.**

The MCT1 knockdown was achieved by short hairpin RNA (shRNA). ATDC5 cells were transfected with plasmids with shRNA targeting the MCT1 or negative control using Lipo2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc., Massachusetts, USA) according to the manufacturer’s protocol. PFKFB3 cDNA was shuttled into plenti4 vector (Invitrogen; Thermo Fisher Scientific, Inc., Massachusetts, USA) and transfected into ATDC5 cells using Lipo2000. All the shRNAs and cDNA were purchased from Gene-Pharma Co. (Shanghai, China). 48 h after transfection, cells were harvested for further analysis.

**Cell viability**

The cell viability was determined by MTT assay. Briefly, cell culture was replaced with MTT solution (0.5 mg/ml) and incubated for another 4 h after treatment. Dimethyl sulfoxide (DMSO) was used to dissolve the formazan crystals. The value was measured at 570 nm with a microplate reader (TECAN Infinite 200 PRO, Mannedorf, Switzerland).

**RNA isolation and real-time quantitative PCR**

Total RNA were extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and applied to synthesized cDNA by Quantscript RT Kit (TIANGEN Biotech, Beijing, China). All protocols were performed according to the manufacturer’s instructions. Quantitative RT-PCR was carried out using SYBR Green reagent (TIANGEN Biotech, Beijing, China). Data was processed according to \( 2^{-\Delta\Delta C_T} \) method. The primer sequences were referred to previous reports.

**Western blotting**

Cells were lysed with RIPA lysis buffer with protease inhibitor. After centrifugation, the total protein was collected and quantified with BCA.
protein assay. Equal amount of total protein was electrophoresed and transferred to PVDF membrane. After blocking, the membranes were incubated with primary antibodies and secondary antibodies. Finally, the visualization of protein was accomplished by enhanced chemiluminescence reaction (ECL), and analyzed using ImageJ software (Version 1.5, NIH Image, Bethesda, MD). The primary antibodies included: MCT1 (dilution 1:1,000; NO. 85680S; Cell Signaling Technology, Inc., Beverly, MA, USA), PFKFB3 (dilution 1:1,000; NO. 13123; Cell Signaling Technology, Inc., Beverly, MA, USA), iNOS (dilution 1:1,000; NO. 13120; Cell Signaling Technology, Inc., Beverly, MA, USA), Cox-2 (dilution 1:1,000; NO. 12182; Cell Signaling Technology, Inc., Beverly, MA, USA), NOX-4 (1:1,000; ab133303; Abcam, Boston, MA, USA).

**ELISA assay**

The concentrations of cytokines in culture medium were measured by ELISA kits (R&D Systems, Abingdon, UK) according to the protocols.

**ROS detection**

ROS generation was tested by 2,7-dichlorofluorescein diacetate (DCFH-DA) (Nanjing Jiancheng, Nanjing, China). The fluorescent intensities were analyzed by a flow cytometer (488 nm excitation, 521 nm emission) (Beckman Coulter, Inc. Fullerton, CA, USA).

**Statistical analysis**

Data were presented as the mean ± SD. Statistical analyses were performed using SPSS software (Version 18.0, SPSS Inc., Chicago, US). *p* < 0.05 was considered as statistically significant.
Results

MCT1 was increased in LPS-treated ATDC5 cells

ATDC5 cells were treated by LPS (final concentration of 0, 2.5, 5, and 10 μg/ml) and the level of MCT1 expression was measured. The results indicated the transcription (Fig. 1A) and expression (Fig. 1B) of MCT1 was markedly increased in LPS-treated ATDC5 cells in a dose dependent manner. The results demonstrated that MCT1 was increased in LPS-treated ATDC5 cells. LPS at a dose of 5 μg/ml that administrated into ATDC5 cells was used in the following experiments.

MCT1 knockdown improved the survival of LPS-treated ATDC5 cells

To evaluate the effect of MCT1 on ATDC5 cell survival, ATDC5 cells were transfected with MCT1 shRNAs or negative control to silence Mct1, and then treated with LPS (5 μg/ml). The results showed the successful transfection of MCT1 knockdown through qRT-PCR (Fig. 2A) and western blotting (Fig. 2B) in LPS-treated ATDC5 cells. Besides, LPS treatment suppressed the viability of ATDC5 cells, which was also reversed by MCT1 knockdown (Fig. 2C). These results indicated that MCT1 knockdown improved the survival of LPS-treated ATDC5 cells.

MCT1 knockdown alleviated LPS-induced inflammatory response in ATDC5 cells

Furthermore, the levels of IL-1β, IL-6, IL-8 and TNF-α were detected by RT-PCR and ELISA assays. The results showed that LPS significantly increased the transcription (Fig. 3A) and secretion (Fig. 3B) of IL-1β, IL-6, IL-8 and TNF-α in ATDC5 cells. However, MCT1 knockdown inhibited the transcription and secretion of these cytokines. The results indicated that MCT1 knockdown alleviated LPS-induced inflammatory response in ATDC5 cells.

MCT1 knockdown alleviated LPS-induced oxidative stress response in ATDC5 cells

Oxidative stress response was associated with inflammatory reaction. Here, the production of ROS was increased in LPS-stimulated ATDC5 cells, which were inhibited by MCT1 knockdown (Fig. 4A). Besides, the transcription levels of iNOS, COX-2 and NOX-4 were increased in LPS-stimulated ATDC5 cells, which were inhibited by MCT1 knockdown (Fig. 4B). The protein expression analyzed by Western blotting showed the consistent results (Fig. 4C). These above results suggested that MCT1 knockdown alleviated LPS-induced oxidative stress in ATDC5 cells.

MCT1 knockdown inhibited the expression of PFKFB3 in LPS-treated ATDC5 cells

A previous research indicated that MCT1 promoted PFKFB3 expression in microglia cells, the following study thus analyzed the expression...
of PFKFB3 in LPS-treated ATDC5 cells with MCT1 knockdown. As shown in Fig. 5A, the expression of PFKFB3 was increased in LPS-treated ATDC5 cells, which was then inhibited by MCT1 knockdown. The increased production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNFα) induced by LPS was inhibited by MCT1 knockdown, while PFKFB3 overexpression reversed this effect (Fig. 5B). The LPS-induced oxidative stress responses, including iNOS, COX-2 and NOX-4, was also inhibited by MCT1 knockdown, while PFKFB3 overexpression reversed this effect (Fig. 5C). These results demonstrated that the anti-inflammatory and anti-oxidative stress effects of MCT1 knockdown were reversed by PFKFB3 overexpression in LPS-treated ATDC5 cells.

**Discussion**

Monocarboxylate transporters (MCTs), including 14 different subtypes, are a family of H⁺-linked transporters that carry monocarboxylates. In particular, MCT1, MCT2, MCT3, and MCT4 are reported to transport L-lactate. MCT1 is ubiquitously expressed and considered to maintain the energy metabolism and homeostasis of intracellular pH. Recent studies indicated that MCT1 has other significant effects on cells, such as oxidative energy production, inflammatory responses, cell death and osteoclast differentiation. Yoshimura et al. found that in IL-1β-induced cell death activation of chondrocytes, ATDC5, MCT1 was involved and the cell death was in NO and ROS dependent manner. In MCT1-silenced cells, the activation of NF-κB and production of ROS production were suppressed, which indicated that MCT1 contributes to ROS-dependent NF-κB activation and cell death. Yasuhara’s research confirmed the conclusion, showing that IL-1β induces death through mitochondrial dysfunction in NO and ROS-dependent manner.

In the present study, LPS treated murine chondrogenic ATDC5 cells was used to mimic an inflammatory response during OA. ATDC5 cells exhibit chondrogenic differentiation at a high frequency and is considered as an excellent in vitro cell model for skeletal development. This study indicated that LPS reduced the survival rate and increased MCT1 expression in ATDC5 cells. The level of MCT1 was proportional to the dosage of LPS. MCT1 silence significantly inhibited the LPS-induced ATDC5 cell death. The levels of inflammatory mediators, reactive nitrogen and oxygen species were significantly increased in LPS-stimulated ATDC5 cells, which were all decreased by MCT1-silenced. Taken together, MCT1 contributes to LPS-induced ATDC5 cell injury by regulating inflammatory responses and oxidative stress.

Glycolytic disorder has been demonstrated to be a major cause of OA. The fructose 2,6-bisphosphate (F2, 6BP), a potent stimulator of glycolysis, is synthesized and degraded by PFKFBs, and of which, PFKFB3 is the most studied isofrom. The abnormal expression of PFKFB3 has been found in various pathological conditions. de Oliveira et al. identified 112 differentially expressed genes and 2,896 differential methylation genes in patients with OA and found that PFKFB3 was valuable methylation-based biomarkers for OA. Besides, Zou et al. found that PFKFB3 expression was increased in the synovial tissue from rheumatoid arthritis (RA) patients compared with OA patients, which indicated that PFKFB3 could also be a biomarker for identification of different types of arthritis. MCT1 are also important regulators in glycolysis. Kong et al. reported that MCT1 promoted PFKFB3 expression via hypoxia-inducible factor-1α in LPS-induced BV2 cells, and PFKFB3 overexpression restored the classical activation of BV2. The present research reflected the similar conclusion in OA. In LPS-treated ATDC5 cells, PFKFB3 overexpression reversed the effects of MCT1 silence on the levels of inflammatory mediators and oxidative regulators.

These results indicated that MCT1 silence could ameliorate LPS-induced ATDC5 cell injury via PFKFB3.

In summary, LPS induced the injury of ATDC5 cells and increased the expression of MCT1. MCT1 knockout decreased the expression of inflammatory mediators and oxidative stress mediators induced by LPS through PFKFB3. This study provides a potential target for the prevention or treatment of OA and RA.

**Competing Interests**

The authors state that there are no conflicts of interest to disclose.

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