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Shikonin inhibits inflammatory responses in rabbit chondrocytes and shows chondroprotection in osteoarthritic rabbit knee

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ABSTRACT

Shikonin, a natural product from Lithospermum erythrorhizon, exerts a wide range of anti-inflammatory actions both in vitro and in vivo. Matrix metalloproteinases (MMPs) have long been considered as the major catabolic enzymes involved in osteoarthritis (OA) cartilage erosion. Here, we investigated the anti-inflammatory and effects of shikonin on MMPs in both IL-1β induced rabbit chondrocytes and the experimental rabbit OA model induced by anterior cruciate ligament (ACL) transection and evaluated the potential involvement of nuclear factor kappa B (NF-κB) in the processes. In vitro, rabbit chondrocytes were cultured and pretreated with shikonin (0, 1, 5, 10 μM) for 1 h (h) with or without IL-1β (10 ng/ml) for 24 h. The expression of MMPs (MMP-1, MMP-3 and MMP-13) and tissue inhibitors of metalloproteinase-1 (TIMP-1) at mRNA and protein levels were determined by quantitative real-time PCR and ELISA respectively. NF-κB related signaling molecules were investigated by Western blotting. In vivo study, the effects of shikonin on MMPs and TIMP-1 were determined at the gene level and the cartilage damage was evaluated at the histological level after the rabbits sacrificed. We found that shikonin significantly reversed the elevated expression of MMP-1, MMP-3 and MMP-13 and the reduced expression of TIMP-1 at both gene and protein levels in IL-1β induced chondrocytes. Additionally, the reduction of IκBα and the activation of NF-κB p65 induced by IL-1β were subsided by shikonin in rabbit chondrocytes. In vivo, both the cartilage damage and the elevated expression of MMP-1, MMP-3 and MMP-13 and the decreased expression of TIMP-1 were ameliorated in shikonin intra-articular injection knees compared to vehicle knees. Our findings indicated that shikonin have anti-inflammatory and chondro-protective effects and may be a potential therapeutic agent for the treatment of OA.

1. Introduction

Osteoarthritis (OA) is a complex chronic progressive joint disease and predominantly responsible for pain and disability among elderly individuals [1]. The main characterization of OA is a progressive breakdown of articular cartilage. Normally, extracellular matrix (ECM) of articular chondrocytes maintains a dynamic equilibrium between synthesis and degradation. In OA, the equilibrium of ECM is disrupted and thus resulting in a continuous loss of cartilage tissues [2]. Excess matrix metalloproteinases (MMPs) activity is thought to be one of the factors responsible for this ECM destruction and the imbalance between MMPs and tissue inhibitors of metalloproteinases (TIMPs) which may be a lead cause for cartilage breakdown in the development of OA [3].

Accumulating evidences suggest that inflammation is one of the pivotal contributors to cartilage matrix degradation [4–7]. In patients with OA, synovitis could be detected in both early and advanced stages, and it is associated with the increased cartilage lesion and pain [8–10]. For example, IL-1β, a main pro-inflammatory and pro-catabolic cytokine, has puissant bioactivities in promoting cartilage ECM breakdown by inducing excessive secretion of proteolytic enzymes such as MMPs [11]. It is generally known that nuclear factor kappa B (NF-κB) pathway involves in the pathogenesis of OA and mediates the expression of MMPs [12]. Therefore, there is a great interest in exploring specific inhibitors of NF-κB pathway as a probable therapeutic option aimed at reducing ECM destruction induced by MMPs in OA knee.

Shikonin is a major component of root extracts of a traditional medicinal herb (Lithospermum erythrorhizon). It has various pharmacological properties, such as wound healing [13], anti-tumor [14], antioxidant [15] and anti-inflammatory [16,17] properties. The anti-inflammatory effect of shikonin was blocking NF-κB p65 translocation from cytoplasm to nucleus by accumulating high levels of IκBα protein

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in macrophages [17]. However, the effect of shikonin on the equilibrium between synthesis and degradation of cartilage ECM to IL-1β stimulation or its efficacy in OA treatment has not been authenticated.

The present study was performed to determine the effects of shikonin treatment on osteoarthritis in vitro and in vivo. First, we used cell model of rabbit articular chondrocytes under IL-1β stimulation, which can mimic an inflammatory response in chondrocytes in vitro, to investigate the anti-inflammatory effect of shikonin and its possible mechanism on inflammatory responses in chondrocyte. Second, the chondroprotective effects and pharmacodynamic action of shikonin on cartilage in vivo were assessed by intraarticular injection in anterior cruciate ligament (ACL) transection induced rabbit OA model.

2. Materials and methods

2.1. Reagents

Shikonin and 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazoliumbromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). IL-1β was obtained from Peprotech (Rocky Hill, NJ, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, 0.25% trypsin and collagenase II were purchased from Gibco (Grand Island, NY, USA). The primary antibodies (rabbit anti-MMP-1, rabbit anti-MMP-3, rabbit anti-MMP-13, and rabbit anti-TIMP-1) were purchased from Epitomics (Burlingme, CA, USA). Anti-IκBα, anti-NF-κB p65 and anti-β-actin antibodies were obtained from Cell Signaling (Beverly, MA, USA).

2.2. Isolation and culture of chondrocytes

The approval for the use of the animals in this study was granted by Animal Ethics Committee of Xi'an Jiaotong University. Chondrocytes were obtained from knees of 4 weeks old New Zealand white rabbits under an aseptic condition. The cartilage was washed in PBS with 10% penicillin–streptomycin for five times within 10 min, and then cut into small pieces. The mixture was primarily digested for 30 min by 0.25% Trypsin-Ethylenediaminetetraacetic acid (EDTA) and subsequently with 0.2% collagenase II in Dulbecco's modified Eagle's medium(DMEM) for 2 h enzymatic digestion at 37 °C. The articular chondrocytes were cultured in DMEM containing 10% FBS and seeded onto a 75 cm^2 culture flask at 37 °C in a humidified atmosphere of 5% CO_2–95% air. When cells in a petri dish reached 80–90% confluence, they were passaged for three duplicates, and passages 2–4 chondrocytes were used in our study.

2.3. Assessment of cell viability

Cell toxicity of shikonin to chondrocytes was measured by MTT assay. In 96-well plates, chondrocytes (1×10^4/well) were treated with various concentration of shikonin (0, 1, 5 or 10 μM) for 24 h respectively. Then, MTT solution (5 mg/ml in PBS) was added and incubated for 4 h at 37 °C. After the supernatant was removed, DMSO (150 μL) was added to each well and a microplate reader was used to measure the absorbance at 570 nm (Bio-Rad, Hercules, CA, USA).

2.4. Real-time PCR analysis

After stimulation with shikonin (0, 1, 5 or 10 μM) for 1 h and subsequently co-cultured with or without IL-1β (10 ng/ml) for 24 h, total RNAs from different monolayer cultured chondroyctes were isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. For first strand cDNA, total RNA (1 μg) was used as a template to produce cDNA using a reverse transcription kit (Promega, Madison, WI, USA). The expression profiles of MMPs and TIMP-1 were verified by quantitative real-time PCR using the 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) and SYBR Premix Ex Taq™ (TaKaRa Biotechnology Co, Ltd., Japan). GADPH was used as an endogenous control. The $2^{-\Delta\Delta CT}$ method was used to measure the relative changes in targeted gene expression level. The sense and antisense primers used as follows: for MMP-1, S: 5′-TGTATC GTGTTGCAGCTCATGA-3′, A: 5′-AAAGCCCCAATATCAGTAGAA TGG-3′; for MMP-3, S: 5′-CTGGAGGTTTGATGAGAAGA-3′, A: 5′-CAGTTC ATGC TCGAGATTCC-3′; for MMP-13, S: 5′-AGTAGTTCCAAAGGCTACAACTT GTTT-3′, A: 5′-GGAGTGGTCAAGCCCTAAGGA-3′; for TIMP-1, S: 5′-GC AACTCCGACCTTGTCATC-3′, A: 5′-AGCGTAGGTCTTGGTGAAGC-3′; for GADPH, S: 5′-ATGACAACTCCCTCAAGAT-3′, A: 5′-GATCCACAACGGAT AC ATT-3′.

2.5. ELISA

After treated with shikonin (0, 1, 5 or 10 μM) for 1 h and following co-incubation with or without IL-1β (10 ng/ml) for 24 h, the levels of MMP-1, MMP-3, MMP-13 and TIMP-1 protein in culture medium were assayed with an ELISA kit (R&D, Minneapolis, MN, USA) following the specification.

2.6. Western blot

Chondrocytes were pretreated with shikonin (10 μM) for 1 h, followed with or without IL-1β (10 ng/ml) for 24 h. Cells were washed with ice-cold PBS and lysed on ice with lysis buffer. The protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were saturated and blocked with 5% fat-free milk at room temperature for 1 h, before being incubated with rabbit anti-NF-κB p65, IκBα and β-actin at 4 °C overnight. After extensive washing, the second antibody was added. The detection of specific antibody-antigen complexes were carried out using a chemiluminescence (ECL) Western blotting detection kit (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's protocols.

2.7. Animal design

Twelve New Zealand male rabbits weighing approximately from 2.5 to 3 kg were purchased from the animal center of Xi'an Jiaotong University and experimental protocol was in accordance with the NIH guidelines of laboratory animals. Eight rabbits were used to establish

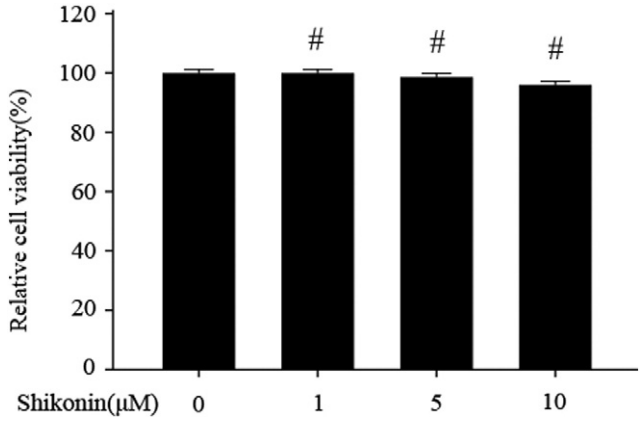

Fig. 1. Effect of shikonin on chondrocytes viability in vitro. The cytotoxicity of shikonin on cells for 24 h was assessed by MTT assay. Cell viability of chondrocytes was not significantly influenced by shikonin at various concentrations (1, 5 or 10 μM) compared with cells incubated with culture medium alone (#$P > 0.05$).