Monotropein accelerates chondrocyte progression in osteoarthritis by alleviating TNF-α induced inflammation through regulation of MAPK/NF-κB signaling pathway

Li Ou, Feng Gao, Min Li, Peifeng Wei

College of Pharmacy, Shaanxi University of Chinese Medicine, Xianyang 712046, China

Received August 27, 2019; Accepted January 7, 2020; Epub February 15, 2020; Published February 28, 2020

Abstract: Background: Osteoarthritis (OA) is a common degenerative musculoskeletal disease in elderly people over 50 years old. Inflammatory response of chondrocytes in OA during treatment vitiated the treatment outcomes. Anti-inflammatory ingredient monotropein has been used in the treatment of OA. However, the molecular mechanism of monotropein in inflammatory response of chondrocytes is unclear. Methods: Cell viability of primary chondrocytes and ATDC5 cells was determined by CCK8 assay. Cell apoptosis was examined by flow cytometry assay. The production of nitric oxide (NO) was detected using Griess reagent. The release of inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-12 (IL-12) was measured by enzyme linked immunosorbent assay (ELISA). The expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) was assessed by qRT-PCR. Protein expression of iNOS, COX-2, matrix metalloproteinases (MMP-1, MMP-3, MMP-13), p-p65, p65, IκB kinase proteins (p-IKK-α/β, IKK-α, IKK-β), inducible nuclear IκB proteins (p-IκB-α, IκB-α), p-ERK1/2, ERK1/2, p-p38 and p38 was analysed by western blot assay. Results: Monotropein exhibited negligible influences on chondrocyte survival. In addition, monotropein alleviated tumor necrosis factor-α (TNF-α) induced inhibition on viability and promotion on apoptosis in chondrocytes. The production of NO, inflammatory cytokines IL-1, IL-6 and IL-12 was enhanced by TNF-α and suppressed gradually by monotropein. Moreover, monotropein attenuated TNF-α induced release of iNOS, COX-2 MMP-1, MMP-3 and MMP-13 in chondrocytes. Besides, monotropein blocked TNF-α induced mitogen-activated protein kinase (MAPK)/NF-κB activation in chondrocytes. Conclusion: Monotropein accelerates chondrocyte progression of in OA by alleviating TNF-α induced inflammation through regulation of MAPK/NF-κB signaling pathway, providing novel therapy strategy for OA.

Keywords: Monotropein, inflammation, chondrocyte, MAPK/NF-κB pathway, OA

Introduction

Osteoarthritis (OA), a frequently diagnosed degenerative musculoskeletal disease particularly in elderly people, is the major cause of movement disorders and has lowered life quality of the patients [1, 2]. It is characterized by oxidative stress, inflammation, effusion, degradation of the articular cartilage, bone remodeling, joint pain, and even disability [3-5]. Currently, the therapeutic strategies against OA emphasized on pain palliation and symptoms reduction by utilizing analgesics and anti-inflammatory medication due to the lack of cartilage regeneration capacity [6, 7]. Therefore, investigation of the pathogenesis is of great significance clinically in developing novel treatments against OA.

Chondrocytes participate in the maintenance of cartilage turnover balance, production of growth factors, regulation of cartilage extracellular matrix (ECM) and response to inflammation [8, 9]. Increased release of inflammatory cytokines, including interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α) and IL-6, encourages the generation of reactive oxygen species (ROS), COX-2, NO and iNOS in chondrocytes, causing cartilage injury [10-12]. In addition, inflammatory cytokines are capable of stimulating matrix metalloproteinases (MMPs, including MMP-1, MMP-3, MMP-13) expression to enhance anabolic activities of chondrocytes by degrading ECM [13, 14]. Recently, inhibition of inflammation of chondrocytes has widely been identified as effective method for OA therapy. For example, artemisate regulated viability,
Monotropein accelerates chondrocyte progression in osteoarthritis

Apoptosis and autophagy of chondrocytes in OA rat models via modulating PI3K/AKT/mTOR signaling pathway [15]. Gastrodin inhibited apoptosis and matrix catabolism mediated by IL-1β induced inflammation to relieve cartilage degeneration in vitro and in vivo [16]. Therefore, the regulatory mechanism of anti-inflammatory medication requires further exploration.

Monotropein, a traditional herbal extracted from the root of *Morinda officinalis* (Rubiaceae), has been used in the prevention and treatment of diabetes, depression, hypertension, Alzheimer disease and OA [17-19]. It possesses antinociceptive, anti-inflammatory, anti-apoptotic and anti-osteoporotic effects by inhibiting iNOS, COX-2, TNF-α and IL-1β associated microRNA expression [20, 21]. Moreover, Zhang et al. considered that monotropein had the ability to accelerate osteoblastic bone formation and alleviate bone loss in ovariectomized mice [22]. However, the function of monotropein and the underlying mechanism during OA therapy are still rarely understood.

In our study, we attempted to reveal the regulatory mechanism of anti-inflammatory agent monotropein on chondrocyte development in OA. As mentioned above, we suggested that monotropein responses to inflammation of chondrocytes by altering NF-κB signaling pathway.

**Materials and methods**

**Cell culture and treatment**

Primary chondrocytes were isolated from 4-day neonatal mice. In brief, neonatal mice were sacrificed and the articular cartilages of knee joints were minced. Then, minced articular cartilages were digested with 0.2% pronase (Sigma Co, St Louis, Mo), 0.25% trypsin and 0.1% collagenase for 1 h and 3 h, respectively. Mouse embryonic tumor cells ATDC5 were purchased from Kanglang (Shanghai, China). The isolated chondrocytes and ATDC5 cells were cultured in Dulbecco modified Eagle medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 0.05% penicillin/streptomycin. Primary chondrocytes and ATDC5 cells were treated with 50 ng/mL TNF-α for 2 h and 50 μM, 100 μM, 200 μM monotropein for 24 h.

**Cell viability assay**

Primary chondrocytes and ATDC5 cells (5000 cells/well) were seeded on 96-well plates for 24 h. Fresh medium containing 50 ng/mL TNF-α was added in each well for 2 h and then fresh medium containing 50 μM, 100 μM, 200 μM monotropein was added in each well for 24 h. After that, the cells were reacted with 10 μL CCK8 reagent (Beyotime, Shanghai, China). The OD value at 450 nm was measured by a spec-
Figure 2. Monotropein alleviated TNF-α mediated inhibition on viability and promotion on apoptosis in chondrocytes. Primary chondrocytes and ATDC5 cells were treated with 50 ng/mL TNF-α for 2 h and 50 μM, 100 μM, 200
Monotropein accelerates chondrocyte progression in osteoarthritis

μM monotropein for 24 h. (A, B) Cell viability of primary chondrocytes (A) and ATDC5 cells (B). (C) Cell apoptosis of primary chondrocytes and ATDC5 cells. (D, E) Caspase 3 activity of primary chondrocytes (D) and ATDC5 cells (E). *P<0.05.

trophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Cell apoptosis assay

Primary chondrocytes and ATDC5 cells (1 × 10^4 cells/well) were seeded on 24-well plates for 24 h. After TNF-α and monotropein treatment, the cells were collected and co-stained with fluorescein isothiocyanate (FITC) tagged Annexin V and propidium iodide (PI) using Apoptosis Detection Kit (Vazyme, Nanjing, China). Cell apoptosis rate was analyzed by BD FACS Canto II flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

NO production

Primary chondrocytes and ATDC5 cells (1 × 10^4 cells/well) were seeded on 24-well plates for 24 h. After TNF-α and monotropein treatment, 50% Griess reagent was added in the cells for 10 min. The OD value at 540 nm was measured by a spectrophotometer.

Enzyme-linked immuno sorbent assay (ELISA)

Primary chondrocytes and ATDC5 cells (5000 cells/well) were seeded on 96-well plate for 24 h. After TNF-α and monotropein treatment, the cells were collected and the release of IL-1, IL-6 and IL-12 was detected using IL-1, IL-6 and IL-12 ELISA kit (Abcam, Cambridge, MA, USA).

Western blot assay

Western blot assay was conducted following the standard protocol. The primary antibodies against iNOS, COX-2, MMP-1, MMP-3, MMP-13, p-p65, p65, p-IKK-α/β, IKK-α, IKK-β, p-IκB-α, IκB-α, p-ERK1/2, ERK1/2, p-p38 and p38 were purchased from Abcam and HRP-conjugated secondary antibody was obtained from Sangon (Shanghai, China).

Quantitative real time polymerase chain reaction (qRT-PCR)

Primary chondrocytes and ATDC5 cells were incubated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to extract total RNA. The cDNA for iNOS and COX-2 was synthesized by All-in-One™ First-Strand cDNA Synthesis Kit (Fuligen, Guangzhou, China). Then, qRT-PCR was performed using SYBR green (Applied Biosystems, Foster City, CA, USA). GAPDH was used as internal reference. The primers for iNOS and COX-2 were listed as follows: iNOS, (Forward, 5’-AATGGCAACATCGGCTCCCTACT-3’; Reverse, 5’-GCTGTGTCAGACAGATCT-CTC-3’); COX-2, (Forward, 5’-GGAGAGACTATCAGAAGTCTCGAACTC-3’; Reverse, 5’-ATGGTCAGTAGACTTTTACA-3’); GAPDH, (Forward, 5’-AGGGTCCGTGGAAAGGATTTG-3’; Reverse, 5’-GGGGGGATTTG-ATGCAACA-3’).

 Luciferase reporter assay

Primary chondrocytes and ATDC5 cells were co-transfected with iNOS-luc, NF-κB-luc and TK-luc plasmids using Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA). After 24 h of transfection, the cells were treated with 50 ng/mL TNF-α for 2 h and 50 μM, 100 μM, 200 μM monotropein for 24 h. Then, luciferase activity was detected using the Promega luciferase assay system (Promega, WI, USA).

Statistical analysis

All the data were presented as means ± standard deviation (SD). Statistical analysis was carried out using SPSS 21.0 software (Chicago, IL, USA) and GraphPad Prism 7 (GraphPad Inc. San Diego, CA, USA). P value less than 0.05 (P<0.05) was considered as statistically significant.

Results

Monotropein displayed limited influences on chondrocyte survival

The effects of monotropein on chondrocyte survival were assessed by CCK8 assay. Cell viability remained unchanged both in primary chondrocytes and ATDC5 cells after monotropein treatment at low concentrations (0 μM, 5 μM, 10 μM, 50 μM, 100 μM, 200 μM) (Figure 1A, 1B). However, monotropein exerted inhibitory function slightly on chondrocyte survival at relatively high concentration (400 μM, 800 μM). Therefore, we concluded that mono-
Monotropein accelerates chondrocyte progression in osteoarthritis

A primary chondrocytes

B ATDC5

C primary chondrocytes

D ATDC5

E primary chondrocytes

F ATDC5

G primary chondrocytes

H ATDC5

Monotropein accelerates chondrocyte progression in osteoarthritis

Figure 3. Monotropein suppressed TNF-α induced release of NO and pro-inflammatory cytokines in chondrocytes. Primary chondrocytes and ATDC5 cells were treated with 50 ng/mL TNF-α for 2 h and 50 μM, 100 μM, 200 μM monotropein for 24 h. (A, B) The production of NO in primary chondrocytes (A) and ATDC5 cells (B). (C, D) The release of IL-1 in primary chondrocytes (C) and ATDC5 cells (D). (E, F) The release of IL-6 in primary chondrocytes (E) and ATDC5 cells (F). (G, H) The release of IL-12 in primary chondrocytes (G) and ATDC5 cells (H). *P<0.05.

Monotropein presented negligible influence on chondrocyte growth.

Monotropein abrogated TNF-α induced inhibition on viability and promotion on apoptosis in chondrocytes

We detected the influences of monotropein on cell proliferation and apoptosis in chondrocytes following TNF-α treatment by CCK8, flow cytometry and Western blot, respectively. As illustrated in Figure 2A, 2B, cell proliferation was inhibited by TNF-α and monotropein reversed the inhibitory effect. By contrast, cell apoptosis rate was enhanced by TNF-α and reduced by monotropein in a dose-dependent manner (Figure 2C). Meanwhile, monotropein reversed TNF-α induced promotive effect on caspase 3 activity (Figure 2D, 2E). Taken together, monotropein relieved TNF-α mediated inhibition on growth and promotion on apoptosis in chondrocytes.

Monotropein attenuated TNF-α induced release of NO and pro-inflammatory cytokines in chondrocytes

Whether monotropein regulates cell progression in chondrocytes after TNF-α treatment by intervening inflammatory response was evaluated by measuring the release of NO and pro-inflammatory cytokines. We observed that monotropein reduced the production of NO in primary chondrocytes and ATDC5 cells after TNF-α treatment (Figure 3A, 3B). More importantly, the release of pro-inflammatory cytokine IL-1 (Figure 3C, 3D), IL-6 (Figure 3E, 3F), IL-12 (Figure 3G, 3H) was up-regulated by TNF-α and down-regulated gradually by monotropein of different concentrations (50 μM, 100 μM, 200 μM). These findings demonstrated that TNF-α could stimulate inflammation and monotropein could prevent chondrocytes from inflammatory response.

Monotropein neutralized TNF-α induced inflammation and ECM degradation in chondrocytes

It is well acknowledged that anti-inflammatory medicine could protect chondrocytes by reducing inflammation and ECM degradation. To confirm the function of monotropein in chondrocyte protection, Western blot, qRT-PCR and luciferase reporter assay were conducted. As displayed in Figure 4A, 4B, the expression of inflammation related protein iNOS and COX-2 induced by TNF-α were decreased after monotropein treatment. Consistently, the expression of iNOS and COX-2 mRNA exhibited the same trends (Figure 4C, 4D). In addition, monotropein suppressed the luciferase activity after TNF-α treatment in chondrocytes transiently co-transfected with iNOS-luc and TK-luc (Figure 4E, 4F). Similarly, the expression levels of matrix degraded enzyme MMP-1, MMP-3 and MMP-13 were elevated by TNF-α. However, the elevation was inversed by monotropein in a concentration dependent manner (Figure 4G, 4H). Collectively, monotropein alleviated TNF-α induced inflammation and ECM degradation in chondrocytes.

Monotropein counteracted NF-κB activation induced by TNF-α in chondrocytes

Previous studies have validated that inflammatory response is mainly affected by the activation of NF-κB pathway. As presented in Figure 5A, 5B, monotropein inhibited TNF-α induced p-p65 expression in chondrocytes. Moreover, luciferase reporter assay revealed that monotropein counteracted TNF-α induced iNOS promoter activity of chondrocytes (Figure 5C, 5D). Altogether, monotropein protected chondrocytes by suppressing NF-κB activation.

Monotropein blocked TNF-α induced activation of the IKK/MAPK pathway

Subsequently, we further explored the regulatory mechanism of monotropein on chondrocytes. As shown in Figure 6A, 6B, protein expression of p-IKK-α/β was up-regulated by TNF-α and down-regulated by monotropein, implicating that monotropein reversed the activation of IKK pathway mediated by TNF-α. Besides, we noticed that IκB-α protein level was inhibited by TNF-α. However, monotropein rescued the suppressive effects induced by...
Monotropein accelerates chondrocyte progression in osteoarthritis

Figure 4. Monotropein inhibited TNF-α induced inflammatory response and extracellular matrix degradation in chondrocytes. Primary chondrocytes and ATDC5 cells were treated with 50 ng/mL TNF-α for 2 h and 50 μM, 100 μM, 200 μM monotropein for 24 h. (A, B) Relative protein expression of iNOS, COX-2 in primary chondrocytes (A) and ATDC5 cells (B). (C, D) Relative expression of iNOS, COX-2 mRNA in primary chondrocytes (C) and ATDC5 cells (D). (E, F) Primary chondrocytes and ATDC5 cells were transiently co-transfected with iNOS-luc and TK-luc for 48 h after TNF-α and monotropein treatment. Luciferase activity of primary chondrocytes (E) and ATDC5 cells (F). (G, H) Relative protein expression of MMP-1, MMP-3, MMP-13 in primary chondrocytes (G) and ATDC5 cells (H). *P<0.05.

Figure 5. Monotropein blocked TNF-α induced NF-κB activation in chondrocytes. Primary chondrocytes and ATDC5 cells were treated with 50 ng/mL TNF-α for 2 h and 50 μM, 100 μM, 200 μM monotropein for 24 h. (A, B) Relative protein expression of p-p65 in primary chondrocytes (A) and ATDC5 cells (B). (C, D) Luciferase activity of primary chondrocytes (C) and ATDC5 cells (D) transiently co-transfected with iNOS-luc and TK-luc for 48 h. *P<0.05.

TNF-α on IκB-α protein generation. Similarly, increased expressions of p-ERK1/2 and p-p38 were appeared after TNF-α stimulation and then decreased by monotropein treatment (Figure 6C, 6D). All the data disclosed that monotropein participated in chondrocytes regulation by blocking TNF-α induced activation of IKK/MAPK pathway.

Discussion

Inflammatory response of chondrocytes in joint tissue during OA progression process comprises the release of cytokine, activation of synoviocytes and cellular infiltration [23, 24]. Reducing inflammation of chondrocytes by genetic engineering and anti-inflammatory agents is presented as a promising strategy for OA therapy [25]. For example, long non-coding RNA MALAT-1 protected articular chondrocytes from IL-1β induced inflammation by regulating the JNK signaling pathway [26]. Similarly, microRNA-93 was reported to induce apoptosis and inflammation of chondrocytes by regulation of Toll-like receptor 4 (TLR4)/nuclear factor-kappa...
Monotropein accelerates chondrocyte progression in osteoarthritis

Anti-inflammatory components geniposide and tenuigenin attenuated inflammation and apoptosis induced by IL-1β to protect rat chondrocytes by the (NF-κB) signaling pathway [27].

Figure 6. Monotropein inhibited TNF-α induced activation of the MAPK pathway. Primary chondrocytes and ATDC5 cells were treated with 50 ng/mL TNF-α for 2 h and 50 μM, 100 μM, 200 μM monotropein for 24 h. (A, B) Relative protein expression of p-IKK-α/β, IKK-α, IKK-β, p-IkB-α, IkB-α in primary chondrocytes (A) and ATDC5 cells (B). (C, D) Relative protein expression of p-ERK1/2, ERK1/2, p-p38, p38 in primary chondrocytes (C) and ATDC5 cells (D). *P<0.05.
Monotropein accelerates chondrocyte progression in osteoarthritis

PI3K/Akt/NF-κB signaling pathway [28, 29]. However, the underlying biological mechanism of anti-inflammatory options is still obscure.

Monotropein is natural iridoid glycoside with mutiple bioactive effects, such as acceleration of osteogenesis, anti-inflammatory and antioxidant in colitis [30-32]. Also, monotropein could benefit wound healing through facilitating angiogenesis and hindering autophagy mediated by oxidative stress in endothelial progenitor cells [33]. In addition, monotropein has the potential to inhibit bone resorption and stimulate bone formation; therefore, monotropein has been used for the prevention and therapy of sciatic neurectomy induced osteoporosis [34]. Likewise, monotropein was clarified to protect osteoblast from inflammatory impairment and LPS-induced bone loss in ovariectomized mice by inactivation of NF-κB pathway [35]. However, the influences of monotropein on the inflammatory response to chondrocyte progression in OA are unknown.

In our present study, we discovered that monotropein exhibited negligible influence on chondrocyte growth at proper concentration. Obviously, TNF-α treatment inhibited cell growth and accelerated apoptosis of chondrocytes. However, monotropein reversed the trends in a dose-dependent manner, indicating that monotropein might exert protective effects on chondrocyte progression. In addition, the productions of NO, IL-1, IL-6, IL-12 were up-regulated after TNF-α addition whereas down-regulated after monotropein treatment, implying that monotropein relieved TNF-α induced inflammatory response. What’s more, the expression levels of iNOS and COX-2 were boosted due to TNF-α induced inflammation and reduced by monotropein. Consistently, monotropein restored TNF-α induced promotion on MMP-1, MMP-3 and MMP-13 protein expression. We suggest that monotropein protected chondrocytes by inhibition of inflammation and ECM degradation. The regulatory mechanism of monotropein on chondrocyte progression was further evaluated by detecting protein levels of p-p65, p-IKK-α/β, IκB-α, p-ERK1/2 and p-p38. The results revealed that monotropein blocked TNF-α induced activation of MAPK/NF-κB signaling pathway.

In conclusion, we demonstrated that monotropein protected chondrocytes in OA by interrupting TNF-α induced inflammation through regulation of MAPK/NF-κB signaling pathway. Our study may provide a guidance on anti-inflammatory therapy of OA.

Acknowledgements

The National Natural Science Foundation of China (Grant no. 81903877). Shaanxi University of Traditional Chinese Medicine Innovation Team Project (Grant no. 2019-QN02).

Disclosure of conflict of interest

None.

Address correspondence to: Li Ou, College of Pharmacy, Shaanxi University of Chinese Medicine, Century Avenue, Xianyang 712046, Shaanxi Province, China. Tel: +86-13891472452; E-mail: uqihewengwaf@163.com

References

[8] Wieland HA, Michaelis M, Kirschbaum BJ and Rudolphi KA. Osteoarthritis—an untreatable dis-
Monotropein accelerates chondrocyte progression in osteoarthritis


Monotropein accelerates chondrocyte progression in osteoarthritis


