Ginkgolide B exerts anti-inflammatory and chondroprotective activity in LPS-induced chondrocytes

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Abstract

Background. Osteoarthritis (OA) is one of degenerative and chronic diseases of articular joints. Articular cartilage is an avascular tissue, and its primary cellular component is chondrocytes. The main characteristic of OA is non-classic inflammation and cartilage degeneration. Ginkgolide B (GB) is a component of *Ginkgo biloba* which has diverse bioactivities.

Objectives. The present study uses an in vitro experimental model to detect the underlying anti-inflammatory and chondroprotective effects of GB and provides a new way for future clinical therapy of OA.

Material and methods. Rat chondrocytes were isolated, cultured and treated with 1 µg/mL lipopolysaccharide (LPS) and/or different concentrations of GB. Cell Counting Kit-8 (CCK-8) was used to test the cell viability of chondrocytes, and chondrocytes apoptosis was detected using a cell apoptosis kit. Collagen-II and aggrecan expression were detected by immunohistochemistry. Relative expression of genes was detected by real-time PCR and western blot.

Results. Ginkgolide B did not inhibit chondrocyte proliferation, and ginkgolide B inhibited LPS induced matrix-degradation in chondrocytes. Ginkgolide B also reversed LPS-induced collagen-II and aggrecan decreased in chondrocytes via upregulated synthesis-related gene expression and downregulated matrix-degrading enzyme gene expression. Furthermore, we found that ginkgolide B significantly inhibited LPS-induced MAPK pathway activation.

Conclusions. The results of our study suggest that ginkgolide B exerted anti-inflammatory and chondroprotective effects in LPS-induced chondrocytes, and might be an underlying therapy for OA afterwards.

Key words: osteoarthritis, anti-inflammatory, chondrocyte, ginkgolide B

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Introduction

Osteoarthritis (OA) is a chronic and degenerative disease of the articular joints. It is thought to cause pain and functional disability in the adult population.¹ According to a report, in America alone, 13% of women and 10% of men over the age of 60 have suffered from knee OA.²

Articular cartilage is an avascular tissue, and its primary cellular component is chondrocytes. Under normal physiological conditions, chondrocytes produce adequate extracellular matrix (ECM) for maintaining cartilage structure and function.³ However, OA cartilage is characterized by a lack of proteoglycans and collagen, which are the main components of ECM. Aggrecan and collagen is degraded by aggrecanases or matrix metalloproteinases (MMPs) in OA cartilage.⁴ Furthermore, in degenerative cartilage, there is evidence of cell death in chondrocytes, which will enhance the degenerative course.⁵

Due to the limited regenerative ability of articular cartilage, OA is one of the most challenging joint diseases. Current pharmacologic treatments include analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs). These treatments provide symptomatic relief, however, they have no effect on OA disease prevention or modification, and substantial side effects of these treatments such as gastrointestinal, renal, and cardiovascular diseases are cause for concern, especially in long-term use.⁶ Therefore, alternative strategies, including traditional Chinese medicine therapy, are becoming prevalent for their clinical curative effect without toxic side effects.

Ginkgolide B (GB) is a component of the ginkgo leaf, which has diverse bioactivities.⁷ As a traditional Chinese drug, ginkgo leaf has commonly been used for treating asthma, cough and enuresis for a long time.⁸ Previous studies have reported that GB has beneficial biological effects on the protection of endothelial cells.⁹ In vitro and in vivo experiments have also shown that GB exerts an anti-inflammatory effect on different cells.^{10–12} However, the effects and underlying mechanism of GB in OA have not yet been reported. To elucidate this question, the present study uses an in vitro experimental model to investigate the underlying chondroprotective and anti-inflammatory efficacy of GB and provides a new way for future clinical therapy of OA.

Material and methods

Reagents

GB was purchased from Sigma-Aldrich (St. Louis, USA) and was dissolved in dimethyl sulfoxide (DMSO) to 100 mM. The working concentration of GB is 50 and 100 μ M.

Chondrocyte isolation and cell culture

Cell collection was approved by the Zhejiang University Animal Experimental Ethics Committee. Ten Sprague Dawley rats (6 weeks old) were euthanized by abdominally injecting a lethal dose of pentobarbital sodium, and then the hip cartilage was isolated. The cartilage was digested at 37°C with 0.2% collagenase for 4 h. After filtration, chondrocytes were cultured in α -MEM (Hyclone, Tauranga, New Zealand) medium, mixed with 10% fetal bovine serum (FBS) (Gibco, Thermo-Fisher Scientific, Waltham, USA) and antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin) (Sigma). Chondrocytes were used within 3 passages.

Measurement of cell viability

Cell viability was measured using a Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). 1×10^4 /mL cells were seeded onto 96-well plates and cultured with various concentrations of GB for 24 h after adherence. At the specified time point, the culture medium was discarded and phosphate buffered saline (PBS) was used to wash the cells. After incubation with CCK-8 solution at 37°C for 2.5 h, the optical density (OD) of the wells at 450 nm was measured using a microplate reader (Thermo Electron Corp., Waltham, USA). The cell viability to control was calculated using the following equation:

$$\text{cell viability to control (\%)} = \text{OD}_{\text{drug-treated group}} / \text{OD}_{\text{control group}}$$

Apoptosis analysis

Chondrocyte apoptosis was detected using a Cell Apoptosis Kit (Becton Dickinson, Franklin Lakes, USA). Chondrocytes were cultured with various concentrations of GB and/or 1 μ g/mL lipopolysaccharide (LPS) for 24 h. Then the cells were washed with cold PBS and resuspended with $\times 1$ annexin-binding buffer. After that, all cells were stained with propidium iodide and fluorescein isothiocyanate (FITC). The apoptosis rate was measured by flow cytometry (FCM). Apoptotic events were indicated as FITC+/PI- (Q2 quadrant in flow cytometry (FCM) figure.

Gene expression

Chondrocytes were incubated with different doses of GB and 1 μ g/mL LPS for 24 h. Total RNA was isolated by the Total RNA Miniprepkit (Axygen Scientific, Corning Inc., Corning, USA). A reverse transcription kit (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, USA) was used to synthesize cDNA using the reverse transcriptional method. mRNA expression levels were evaluated by real-time PCR (Reagent Kit: SYBR® Premix ex Taq™, TliRNase H Plus, Takara, Tokyo, Japan) according to the manufacturer's instructions. The PCR setting was as follows: 40 cycles at

Table 1. Sequences of primers used in real-time PCR

Gene	Primer sequences (5'-3')	
Collagen II	forward	GGCCAGGATGCCCGAAAATTA
	reverse	ACCCCTCTCTCCCTTGTCAC
Aggrecan	forward	CAGATGGCACCTCCGATAC
	reverse	GACACACCTCGGAAGCAGAA
MMP3	forward	TTTGCCGCTCTTCCATCC
	reverse	GCATCGATCTTCTGGACGGT
MMP13	forward	ACCATCCTGTGACTCTTGCG
	reverse	TTCACCCACATCAGGCACTC
ADAMTS-4	forward	ACCGATTACGACCTTTGGG
	reverse	CCGACTCCGGATCTCCATTG
ADAMTS-5	forward	CCGAACGAGTTTACGGGGAT
	reverse	TGTGCGTCGCCTAGAACTAC
IL-1	forward	ATCAGCACCTCACAGCTTCC
	reverse	TCTCTCCCGATGAGTAGGC
IL-6	forward	CACTTCACAAGTCGGAGGCT
	reverse	TCTGACAGTGCATCATCGCT
TNF- α	forward	GGCTTTCGGAACCTCACTGGA
	reverse	GGGAACAGTCTGGGAAGCTC
GAPDH	forward	TGCCACTCAGAAGACTGTGG
	reverse	TTCAGCTCTGGGATGACCTT

MMP3 – matrix metalloproteinase 3; MMP13 – matrix metalloproteinase 13; IL-1 – interleukin 1; IL-6 – interleukin 6; TNF- α – tumor necrosis factor alpha; GAPDH – glyceraldehyde 3-phosphate dehydrogenase.

95°C for 5 s and 60°C for 34 s. The primers were designed and selected using BLAST. The primer sequences are listed in Table 1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control.

Western blot analysis

For aggrecan and collagen II protein assay, the cells were treated with different doses of GB and/or 1 μ g/mL LPS for 5 or 8 days. For mitogen-activated protein kinase (MAPK) pathway protein assay, the cells were treated with different doses of GB and/or 1 μ g/mL LPS for 24 h. At specific time points, the cells were washed with $\times 1$ PBS, then total protein was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer. The protein concentration was quantified using a bicinchoninic acid (BCA) Protein Assay Kit, 20 μ g protein (each sample) was loaded into gel, and separated by 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), then transferred to 0.22- μ m PVDF (polyvinylidene difluoride) membranes (Millipore, Merck, Billerica, USA). The membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) and 5% nonfat milk powder and then incubated overnight with MAPK pathway-related antibodies, such as p-ERK, total-ERK, p-JNK, total-JNK, p-p38 and total-p38, and GAPDH overnight at 4°C (1:1000 dilution, Cell Signaling Technology, Danvers, USA). After 3 washes with TBST, the membranes were incubated with anti-rabbit IgG (1:5000 dilution, Cell Signaling Technology) at room temperature for 1 h. After 3 washes with TBST, the membranes were incubated in enhanced chemiluminescence (ECL)

solution (Amersham Life Science, GE Healthcare Life Sciences, Little Chalfont, United Kingdom). GAPDH was used as the house-keeping gene.

Immunohistochemistry staining

Three 10^4 /mL cells were seeded in 24-well plates and cultured with different doses of GB and/or 1 μ g/mL LPS for 5 or 8 days. Cells were fixed with 4% paraformaldehyde before making cell slides. After fixation, the cells were treated with 0.1% Triton X-100 for 10 min, and blocked with 2% bovine serum albumin (Sigma-Aldrich, St. Louis, USA) for 1 h. Then, cells slides were incubated with an anti-collagen II antibody (1:200 dilution, Abcam, Cambridge, UK) and anti-aggrecan antibody (1:200 dilution, Abcam, Cambridge, UK) overnight at 4°C. For immunohistochemistry, cell slides were incubated with secondary antibody (1:200 dilution, Dako, Agilent Technologies, Santa Clara, USA), followed by color development with diaminobenzidine tetrahydrochloride (DAB, Dako). We used an inverted microscope microscopy (Olympus Life Science, Shinjuku, Tokyo, Japan) for observation and imaging. The integral optical density (IOD) of each picture was measured using the Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, USA).

Statistical analysis

SPSS 19.0 (IBM Corp., Armonk, USA) was used for the statistical analysis. The data is expressed as mean $\pm$ SD. The significance of differences was assessed with one-way analysis of variance (ANOVA), followed by Duncan's post

hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cell viability assay

To study the potential cytotoxicity of GB, we measured chondrocyte viability. After incubating with different concentrations of GB for 24 h, we used CCK-8 to assay the cell

Fig. 1. Cell viability assay of chondrocytes

10000/mL cells were seeded onto 96-well plates and cultured with various concentrations of ginkgolide B for 24 h after adherence. Then, we used a CCK-8 to test the cell viability of the chondrocytes.

viability of chondrocytes. As shown in Fig. 1, GB did not inhibit chondrocyte proliferation at concentrations of 50 and 100 μM.

Ginkgolide B protected chondrocytes from inflammation-induced apoptosis

We examined the apoptosis rate of chondrocytes after treating with 1 μg/mL LPS for 24 h. Figure 2A shows LPS-induced chondrocyte apoptosis, while GB protected chondrocytes from inflammation-induced apoptosis at concentrations of 50 and 100 μM ($p < 0.05$) (Fig. 2B).

Ginkgolide B inhibited LPS-induced matrix-degradation in chondrocytes

Chondrocytes were incubated with 1 μg/mL LPS with or without GB (50 or 100 μM) for 5 or 8 days. After that, we detected extracellular matrix content by cell immunohistochemistry. Our results indicate that the expression of collagen-II and aggrecan increased over time in chondrocytes, while LPS strikingly reduced the aggrecan and collagen-II content ($p < 0.05$) (Fig. 3A). Immunohistochemistry staining showed GB significantly suppressed the degradation of aggrecan and collagen-II compared to the LPS group ($p < 0.05$) (Fig. 3A). The quantification of IOD also indicated that the GB groups gained more aggrecan and collagen-II staining (Fig. 3B,C).

After being incubated with LPS and various concentrations of GB for 5 or 8 days, we also used western blot to measure the collagen-II and aggrecan protein content. The result was similar to the immunohistochemistry staining. As shown in Fig. 4, LPS-induced collagen-II and aggrecan protein down-expression, and GB significantly attenuated the collagen-II and aggrecan loss. This effect was more obvious on day 8 ($p < 0.01$) (Fig. 4).

Fig. 2. Apoptosis assay of chondrocytes

A – after stimulation with or without 1 μg/mL LPS and different doses of ginkgolide B for 24 h, cells were stained with annexin V-FITC and propidium iodide and the apoptosis rate was measured via flow cytometry; B – the apoptosis rate (Q2) was calculated and compared; data is presented as the mean ± SD; *** indicates $p < 0.001$; ** indicates $p < 0.01$; * indicates $p < 0.05$ compared to the control group.

Fig. 3. Immunohistochemistry staining of chondrocytes

A – 30000/mL cells were seeded in 24-well plates and cultured with different doses of ginkgolide B and/or 1 g/mL LPS for 5 or 8 days, then cells were fixed with 4% paraformaldehyde and immunohistochemistry staining of aggrecan and collagen-II was performed; B, C – results of immunohistochemistry staining were quantified by integrated optical density (IOD); data is presented as the mean ± SD; *** indicates $p < 0.001$; ** indicates $p < 0.01$ compared to the control group.

Fig. 4. Matrix-related protein expression of chondrocytes

We used western blot to measure aggrecan and collagen-II protein expression of chondrocytes. Cells were cultured with different doses of ginkgolide B and/or 1 μg/mL LPS for 5 or 8 days, then the total protein of cells was extracted using RIPA lysis buffer and western blot was performed. A, C – expression of collagen-II and aggrecan at 5 and 8 days; B, D – GAPDH was used as the house-keeping gene; data is presented as the mean ± SD; *** indicates $p < 0.001$; ** indicates $p < 0.01$; * indicates $p < 0.05$ compared to the control group.

Fig. 5. Gene expression changes in chondrocytes

A–I – chondrocytes were treated with or without 1 µg/mL LPS and various concentrations of ginkgolide B for 24 h; total RNA was isolated, then the gene expression of collagen-II, aggrecan, MMP-13, MMP-3, ADAMTS-5, ADAMTS-4, IL-1, IL-6 and TNF-α were measured by real-time PCR; GAPDH was used as the internal control; data is presented as the mean ± SD; *** indicates $p < 0.001$; ** indicates $p < 0.01$; * indicates $p < 0.05$ compared to the control group.

Ginkgolide B reversed LPS-induced gene expression changes in chondrocytes

Chondrocytes were stimulated with 1 µg/mL LPS and 0, 50 or 100 µM GB, followed by real-time PCR assay. PCR results showed that LPS significantly up-regulated the gene expression of proinflammatory cytokines (IL-1, IL-6 and TNF-α). LPS also down-regulated the gene expression of collagen-II and aggrecan in chondrocytes, which was reversed by GB ($p < 0.01$) (Fig. 5A,B), and GB also inhibited multiple matrix-degrading enzyme (MMP-13, MMP-3, ADAMTS-5 and ADAMTS-4) gene over-expression induced by LPS in chondrocytes ($p < 0.05$) (Fig. 5C–F).

IL-6, TNF-α and IL-1 were downregulated by GB ($p < 0.001$) (Fig. 5G–I), so GB has an anti-inflammatory effect in LPS-induced chondrocytes.

Ginkgolide B inhibited LPS-induced activation of MAPK pathway in chondrocytes

We measured the activation of MAPK pathway-related proteins by western blot to evaluate the potential mechanisms of the effects of GB on LPS-induced chondrocytes. Cells were pretreated with different doses of GB for 2 h, then treated with or without 1 µg/mL LPS for 30 min.

Fig. 6. Effect of ginkgolide B on LPS-induced activation of the MAPK pathway in chondrocytes

A – chondrocytes were pretreated with various concentrations of ginkgolide B for 2 h, then treated with or without 1 μg/mL LPS for 30 min; the expression of MAPK pathway proteins was measured by western blot; B – results were densitometrically quantified as the ratio of p-ERK to total-ERK, p-JNK to total-JNK and p-p38 to total-p38; data is presented as the mean ± SD; *** indicates $p < 0.001$; ** indicates $p < 0.01$; * indicates $p < 0.05$ compared to the control group.

The results showed that 1 μg/mL LPS significantly activated the MAPK pathways by promoting JNK, ERK and P38 protein phosphorylation (Fig. 6), and GB could inhibit the phosphorylation of JNK, ERK and P38 in a dose-dependent manner (Fig. 6). This result indicates the MAPK pathway was involved while GB exerted an anti-inflammatory effect in chondrocytes.

Discussion

Our study indicated that GB exhibited anti-inflammatory effects in chondrocytes for the first time.

Chondrocyte apoptosis plays a key role in the pathogenesis of OA.^{13,14} Chondrocytes from normal donors did not show any apoptosis signs, while chondrocytes isolated from human OA cartilage exhibited multiple morphological evidence of apoptosis.^{15,16} A proper balance between cell death and regeneration is essential for a healthy joint, and apoptosis excess is one of the reasons for tipping this balance.¹⁷ As previously reported, treating chondrocytes with IL-1β could reduce chondrocyte viability and upregulate apoptotic-related proteins, and several anti-inflammatory agents had been believed to exert anti-apoptosis effects in chondrocytes.^{13,18} The dose of GB used in different cells varies from 25–1000 μM, and in our study, we demonstrated that GB exerted anti-inflammation and anti-apoptosis effects in chondrocytes at doses of 50–100 μM.^{7,10,12}

Previous studies have suggested that LPS markedly induces upregulation of genes and the generation of various matrix-degrading enzymes and proinflammatory cytokines, including IL-6, Cox-2, ADAMTS-5, ADAMTS-4, MMP-13 and MMP-3, in chondrocytes.¹⁹ The expression of ADAMTSs and MMPs has been extensively detected in the process of OA. In the expression of MMP-13 associated with OA, as shown in recent studies, osteoarthritis progression was inhibited in MMP-13 knockout mice.²⁰ MMP-3 is a superior indicator of joint destruction that cleaves aggrecan, and the cartilage degradation

is related to the activity of MMP-3 enzymes.^{21–23} Besides MMPs, ADAMTSs are also believed to be involved in the OA process.²⁴ ADAMTS-4 and ADAMTS-5 are the most important aggrecanases among the 20 different ADAMTSs.²⁵ ADAMTS-5 was responsible for driving aggrecan loss in mice, which would result in cartilage damage, and an animal experiment also showed mouse with a knockin aggrecan mutated to have aggrecanase resistance successfully suppressed both meniscectomy-induced and antigen-induced arthritis.^{26,27} The gene expression of both enzymes was upregulated by inflammatory stimuli such as TNF-α and oncostatin M in both human and mouse chondrocytes.^{28,29} Moreover, inflammatory stimuli lead to matrix breakdown in chondrocytes.³⁰ The matrix of chondrocytes, which includes pericellular matrix (PCM) and ECM, conferred mechanical properties of cartilage. Loss of collagen II and proteoglycan significantly reduced chondron's mechanical properties.³¹ Progressive breakdown of cartilage in OA involves proteolysis of both aggrecan and collagen II.³² In our study, we demonstrated that GB could reverse the gene over-expression of MMP-13, MMP-3, ADAMTS-5 and ADAMTS-4 induced by LPS in chondrocytes, and GB also efficiently inhibited the collagen II and aggrecan loss of chondrocytes after stimulation with LPS.

Ginkgolide B is a component of the ginkgo leaf, and Egb761, a commercial product of *Ginkgo biloba* extract, has been used in various diseases such as cardiovascular disease, hearing loss, and Alzheimer's disease.³³ Ginkgolide B was proved to be an effective anti-inflammatory agent in different diseases.^{10,11,34} MAPK is an important pathway that is involved in chondrocyte mechanical stress and inflammation.³⁵ The effect of inhibiting p38 in cartilage was tested in a previous study.³⁶ The results indicated that inhibition of p38 suppressed IL-1-induced MMP-1 and MMP-3 expression and collagen breakdown in cartilage. The role of the ERK pathway had also been investigated in a rabbit osteoarthritis model, and treatment with an ERK inhibitor reduced cartilage lesion size and histological damage.³⁷

The JNK inhibitor SP600125 inhibited collagenase mRNA accumulation in synoviocytes after stimulation with IL-1, and JNK is a critical MAPK pathway for joint arthritis.³⁸ Our western blot results showed that GB inhibited the activation of the MAPK pathway in chondrocytes, thus exerting an anti-inflammation effect, which is consistent with previous reports.^{34,39}

In conclusion, our results show that GB exerted anti-inflammatory and anti-apoptosis effects in chondrocytes. Ginkgolide B may be a potential agent for treatment of OA. However, more research should be done in the future to further study the efficacy and mechanism of GB.

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