Ginsenoside Rb1 inhibits matrix metalloproteinase 13 through down-regulating Notch signaling pathway in osteoarthritis

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Abstract

Mounting evidence suggests that an excess of matrix metalloproteinase-13 (MMP-13) plays an important role in the breakdown of extracellular matrix in osteoarthritis (OA). Here, the effects of ginsenoside Rb1 (GRb1) on the expression of MMP-13 in IL-1 β -induced SW 1353 chondrosarcoma cells and an experimental rat model of OA induced by anterior cruciate ligament transection (ACLT) were investigated. SW1353 chondrosarcoma cells were pretreated with or without GRb1 and Notch signaling pathway inhibitor, DAPT, then were stimulated with IL-1 β . In rats, experimental OA was induced by ACLT. These rats then received intra-articular injections of vehicle, an inhibitor of γ -secretase, DAPT, and/or GRb1. Expression of MMP-13, collagen type II (CII), Notch1, and jagged 1 (JAG1) were verified by western blotting and immunohistochemistry. In addition, levels of MMP-13 mRNA were detected using quantitative real-time PCR. In histological analyses, treatment with DAPT reduced the number of cartilage lesions present and the expressions of MMP-13, CII, Notch1, and JAG1. In addition, treatment with GRb1 was associated with lower levels of Notch1 and JAG1 in both IL-1 β -induced SW1353 chondrosarcoma cells and in the rat OA model. Furthermore, the suppressive effect of GRb1 on MMP-13 was greater than that exhibited by the signaling pathway inhibitor. In conclusion, GRb1 inhibits MMP-13 through down-regulating Notch signaling pathway in OA.

Keywords: Matrix metalloproteinase-13, ginsenoside Rb1, notch signaling pathway, osteoarthritis

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Introduction

Osteoarthritis (OA) is a debilitating disease and has been recognized as the major form of articular disease affecting nearly 80% of people beyond the age of 65 years.¹ No therapy is currently available to completely prevent the initiation or progression of the disease partly due to poor understanding of the mechanisms of the disease pathology. Articular cartilage, an aneural, avascular tissue, which consists of one cell type, the chondrocyte, and the extracellular matrix (ECM), is the main target afflicted by OA. ECM is mainly composed of collagens and aggrecan, of which the former provides tensile strength for the tissue, and the latter, a negatively charged proteoglycan, provides compressive resistant and deforms while absorbing impact loads.² Proteolytic degradation of ECM is the hallmark of osteoarthritic joint lesion. During OA, chondrocytes undergo phenotypic modifications, such as the acquisition of a fibroblast-like morphology, loss of the ability to express collagen II, and increased expression of fetal fibrillar collagen type I. It is precisely because of these changes that matrix degrades and cartilage fails to repair successfully.^{3,4}

Furthermore, chondrocytes and the synovial tissue secrete inflammatory cytokines, such as IL-1 β , TNF- α , which promote chondrocytes to secrete proteases, most notably matrix metalloproteinases (MMPs). MMPs are a family of zinc-containing, calcium-dependent neutral proteases which possess the effective capacity of initiating the cleavage of collagen type II (CII) and aggrecan. Of the MMPs, MMP-13 has the broadest substrate specificity and exhibits the highest activity toward CII, the predominant collagen in cartilage,⁵ which was mediated by various metabolic pathways, like mitogen-activated protein kinases (MAPKs), nuclear factor kappa B (NF- κ B), and Wnt/ β -catenin signaling.

Notch signaling is a highly conserved pathway regulating cell-to-cell interaction required for the determination of cell fate.⁶ In the Notch signaling pathway, there is also a ligand of Notch1, which called jagged 1 (JAG1), and interacts with Notch1. It has been recently reported that the expression of Notch family members in normal and OA cartilage raised the issue of the involvement of this pathway in the physiopathology of OA, especially in the changes that chondrocytes undergo during OA process. It is also

Table 1 Histological score of articular cartilage

	Normal (n = 10)	Vehicle (n = 10)	DAPT (n = 10)	GRb1 (n = 10)
Structural changes	0.77 ± 0.13	4.18 ± 0.27*	3.06 ± 0.25#	2.32 ± 0.35#
Cellular changes	1.18 ± 0.09	2.54 ± 0.19*	2.06 ± 0.14#	1.73 ± 0.11#
Tide mark	0.45 ± 0.08	1.35 ± 0.18*	0.97 ± 0.08#	0.78 ± 0.17#
Total score	1.40 ± 0.30	8.07 ± 0.64*	6.09 ± 0.47#	4.83 ± 0.63#

Values are the mean ± SD. #P < 0.05 compared with vehicle group; *P < 0.05 compared with other groups.

documented that cytokine activation of Notch signaling was dependent on both NF-κB and MAPK pathways.⁷

Ginsenoside Rb1 (GRb1), an active compound, which is isolated from Chinese herb *Panax ginseng*, possesses a variety of biological activities including antioxidant, anti-inflammatory, and antiapoptosis effects.⁸ Previous researches have proved that GRb1 can provide effective therapy for neurodegenerative diseases and exert suppressive effects on intestinal⁹ and cerebral ischemia.¹⁰ Furthermore, GRb1 can protect articular chondrocytes from hydrogen peroxide-induced oxidative stress¹¹ and prevent articular chondrocytes from IL-1 beta-induced inflammation and apoptosis.¹² However, the detail molecular mechanisms of the protection have not reported yet.

In the present study, we examined the protective effects of GRb1 against OA *in vivo* and *in vitro* and explored the underlying mechanisms.

Materials and methods

Cell culture

Cultures of SW 1353 chondrosarcoma cells (Institute of Biochemistry and Cell Biology, Shanghai, China) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco, Grand Island, NY), 2 mmol/L glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Cells were treated with or without IL-1β (Pepro Tech, NJ, United States) at 10 ng/mL for 24 h in the absence or presence of GRb1 (Sigma, Texas, USA) at 20, 40, and 80 µM at the final concentration, respectively. In another set, the cells were stimulated with IL-1β at 10 ng/mL for 24 h after being pretreated with DAPT at 10 µM for 1 h. Then cells were harvested for the further experiments.

Quantitative reverse transcription-polymerase chain reaction (q-PCR)

Total RNA was extracted with the TRIzol reagent, according to manufacturer's protocol (Invitrogen, Shanghai, China). After RNA's sample concentrations and integrity were measured, the reverse transcription was performed using the Rever Tre Ace-a- reverse transcriptase cDNA synthesis kit (TOYOBO, Japan) as recommended by the manufacturer. q-PCR array was performed using an a 7500 Real Time PCR System (Biosystems, Singapore) and SybrGreen PCR (TOYOBO, Japan) following the program: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. A 10-min 72°C extension step completed the

reaction. Expression of β-actin was used as endogenous control. The human MMP-13 primers were 5'-TCCTGATG TGGGTGAATACAATG-3' (upper) and 5'-GCCATCGT GAAGTCTGGTAAAAT-3' (lower). The 2-DDCT method was used to calculate the relative fold changes in mRNA expression.

Western blotting

Total protein extraction was performed using an extraction kit (Beyotime Institute of Biotechnology, Jiangsu, China). Protein concentrations were determined using Enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China). Equal amounts of proteins were separated on an 8 or 10% SDS-polyacrylamide gel and then transferred electrophoretically to a polyvinylidene fluoride membrane. Membranes were blocked with 5% bovine serum albumin for 2 h. They were then incubated with primary polyclonal antibodies at 4°C overnight. Immunodetection was performed using peroxidase-conjugated antibodies and the signal was detected using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology, Jiangsu, China).

Animal experiments

Forty eight-week-old SD rats were obtained from Animal Center of Chongqing Medical University. With the approval of the Institutional Animal Care and Use Committee of Chongqing Medical University (Chongqing, China), 30 rats were received anterior cruciate ligament transection (ACLT) in the right posterior knee joint to duplicate the experimental OA model, following previous description,¹³ then randomly divided into three groups. After four weeks, these rats received weekly intra-articular injection, with or without GRb1 (0.3 mL, 80 µM), Notch inhibitor DAPT (0.3 mL, 10 µM). The rats were sacrificed six weeks after injection. The other 10 rats were used as the normal control.

Histologic evaluations

The femoral condyles were removed, fixed in 4% paraformaldehyde, decalcified, embedded in paraffin, and sliced at 4 µm thickness. The sections were obtained and stained with hematoxylin and eosin. The histologic evaluation was performed by using the Mankin articular cartilage injury method. The Mankin articular cartilage injury scores are composed of cartilage structure scores, cell accounts scores, and Tidemark scores. In this study, the

observers determined the score using the criteria and scoring system formulated by Mankin et al.¹⁴

Immunohistochemistry

Sections were deparaffinized in xylene and rehydrated through descending ethanol series. They were then immersed in 10 mM citric acid monohydrate (pH 6.0) for 10 min and incubated in 0.1% trypsin for 30 min at 37°C. After that, rabbit anti-rat primary polyclonal antibodies against MMP-13, CII, Notch1, and JAG1 were incubated on the slides overnight at 4°C in wet room, then with secondary antibody (goat anti-rabbit, Santa Cruz). The expressions were visualized by Chromogen 3,3'-diaminobenzidine immunolabeling.

Statistical analysis

Each experiment was independently performed three times. All data were expressed as mean $\pm$ standard deviation. Statistical comparisons between groups were performed using one-way analysis of variance followed by Tukey *post hoc* test. Values of $P < 0.05$ were considered to indicate statistical significance.

Results

Effects of GRb1 treatment at different concentrations on MMP-13 and CII expression in IL-1 β -induced human SW1353 cells

As expected, stimulation with IL-1 β resulted in the increase of MMP-13 and decrease of CII. Treatments with 20, 40, and 80 μ M GRb1 were found to inhibit an increase in MMP-13

and a decrease in CII in response to pretreatment with IL-1 β , furthermore the latter concentration produced a more significant effect ($P < 0.05$) (Figure 1). In contrast, stimulations of MMP-13 expression by IL-1 β were inhibited by treatment with 80 μ M GRb1 compared with cells only treated with IL-1 β or cells treated with 20 or 40 μ M GRb1 (Figure 1, $P < 0.05$), while the decreases of CII were opposite (Figure 2). This result is consistent with our previous findings obtained from the *in vitro* study. Therefore, 80 μ M of GRb1 were used in subsequent experiments.

Levels of MMP-13 and CII were observed to significantly increase and decrease separately after stimulation of IL-1 β compared with the control group ($P < 0.05$).

Effects of GRb1 treatment on Notch1 and JAG1 in IL-1 β -induced SW 1353 cells and in cartilage

To examine the effects of GRb1 on the activation of Notch1 and JAG1, cell extracts and cartilages were collected and analyzed using western blotting. Expression levels of these two proteins significantly increased in IL-1 β -induced SW 1353 cells (Figure 2) and in cartilage of vehicle group. Knee joint sections showed a smooth surface; a clear laminar structure; and low-level expressions of MMP-13, Notch1, and JAG1 but a high level of CII in the articular cartilage from the normal group (Figure 3(a)). In contrast, knee joint sections showed a rough surface with some superficial leakage and ulcers and high-level expressions of MMP-13, and Notch1 for the vehicle-treated group, while the expression of CII was at a higher level (Figure 3(a)). Furthermore, GRb1-treated cartilage and cartilage treated with signaling pathway-specific inhibitor showed fewer lesions and MMP-13, and Notch1 but more CII,

Figure 1 Effects of GRb1 on MMP-13 and CII expression. Human SW 1353 chondrosarcoma cells were pretreated with or without GRb1 (20, 40, 80 μ M) for 1 h, then stimulated with 10 ng/mL IL-1 β for 24 h. Expressions of CII (a) and MMP-13 (b) were assayed by using western blot. The data presented are the mean $\pm$ SD. $P < 0.05$, $P < 0.01$ represent protein expression in control or IL-1 β group compared to IL-1 β + GRb1 group. \$ $P < 0.05$, ** $P < 0.01$, * $P < 0.05$ represent the protein expression in IL-1 β + GRb1 group compared to the IL-1 β group. This experiment was repeated at least three times

Figure 2 Effects of GRb1 on the expressions of Notch1 and JAG1 in IL-1 β -induced SW 1353 cells. (a) Data showed that IL-1 β resulted in the increases of Notch1, as well as JAG1, and the decrease of CII. In contrast, levels of Notch1 and JAG1 were down-regulated and CII was up-regulated following treatment with GRb1. (b) Four weeks after ACLT was performed, intra-articular injections of GRb1, a vehicle control, or a pathway inhibitor were made once a week for six weeks. Extracts of resected knee joints were assayed for levels of Notch1 and JAG1 by western blot. The data presented are the mean $\pm$ SD. The concentration of IL-1 β used in this study is 10 ng/mL, and treating for 24 h. Treatment with 80 μ M GRb1 (^{*}) (treating for 1 h) resulted in lower levels of Notch1 and JAG1 compared with the vehicle group ($P < 0.05$). This experiment was repeated at least three times

Figure 3 Effects of GRb1 on Notch1 and JAG1 expressions in cartilage. ACLT was applied to the rats in the right knee to duplicate an experimental OA model. Four weeks later, rats received an intra-articular injection of GRb1, DAPT or vehicle once a week for six weeks. (a) Cartilages of resected knee joints were assayed for the levels of MMP-13, CII, and Notch1 by immunohistochemistry assay. Magnification, 200 $\times$. (b) Cartilages of resected knee joints were assayed for the levels of MMP-13, CII, Notch1, and JAG1 by western blot assay. [#] $P < 0.05$, ^{##} $P < 0.01$ represent the protein expression in normal or vehicle group compared to GRb1 group. ^{*} $P < 0.05$ represents protein expression in GRb1 group compared to vehicle group. This experiment was repeated at least three times. (A color version of this figure is available in the online journal.)

Figure 4 The use of signaling pathway inhibitor and GRb1 to investigate IL-1 β -mediated MMP-13 gene expression. Data are expressed as the mean $\pm$ SD. # P < 0.05 compared with the control group and IL-1 β group. * P < 0.05 compared with the other groups. This experiment was repeated at least three times

compared with vehicle-treated cartilage sections. Data of western blot assays showed that expressions of MMP-13, Notch1, and JAG1 were up-regulated in vehicle group and down-regulated with treatment of GRb1 (Figure 3(b)). Furthermore, the expression of CII was inhibited in cartilages of vehicle group but promoted in cartilages of GRb1 group.

Furthermore, the decreases in expression were statistically significant (P < 0.05). Moreover, stimulations of MMP-13 expressions by IL-1 β and ACLT were inhibited by treatment with GRb1 compared with cells only treated with IL-1 β and cartilages in vehicle group (P < 0.05). The scores of histological staining of articular cartilage sections were presented in Table 1.

Inhibition of MMP-13 by pathway inhibitors and GRb1 in IL-1 β -induced cells and in cartilage

To investigate the *in vitro* effects of GRb1 treatment on the expression of MMP-13 in IL-1 β -induced SW 1353 cells, extracts of IL-1 β -induced cells were assayed for gene expression levels of MMP-13 by q-PCR, and treatment with 80 μ M GRb1 resulted in lower MMP-13 mRNA compared to the IL-1 β -treated groups and vehicle groups (Figure 4, P < 0.05).

Using the experimental *in vivo* model, weekly intra-articular injections of 80 μ M GRb1 and 10 μ M DAPT (a Notch inhibitor) were performed for six weeks. Extracts of resected knee joints were then assayed for levels of MMP-13, JAG1, Notch1, and CII by western blot (Figure 5). ACLT-induced levels of former three targets were found to be significantly reduced by signaling pathway inhibitor compared with the vehicle group, while the last target was

significantly increased (P < 0.05). Furthermore, ACLT-induced expression of MMP-13 exhibited a greater reduction following treatment with GRb1 than with the signaling pathway inhibitor (P < 0.05), consistent with the *in vitro* findings described earlier.

Discussion

OA is a degenerative joint disease and is the most prevalent form of arthritis, of which the major symptom is progressive cartilage break down and eventual complete loss of articular cartilage.^{15,16} The present study showed that GRb1 at the concentration of 80 μ M could significantly decrease the up-regulated MMP-13 and enhance the down-regulated CII in IL-1 β -induced SW 1353 cells and in osteoarthritic cartilage. Moreover, we found that the chondroprotective effect of GRb1 was associated with its suppression of Notch signaling pathway via attenuating Notch1 and JAG1 levels.

It is widely accepted that IL-1 β induced cell apoptosis and activation of MMPs, which was often used to imitate the cartilage damage caused by OA. MMPs have long been recognized for their ability to catalyze the hydrolysis of ECM molecules, including collagens and aggrecans.¹⁷ Among all the MMPs, MMP-13 is a major enzyme that targets cartilage for degradation and is more restricted to connective tissue,¹⁸ which not only targets type II collagen in cartilage for degradation, but also degrades proteoglycan type IV and type IX collagen, osteonectin, and perlecan in cartilage.¹⁹ Little *et al.* showed that global knock out of MMP-13 could prevent articular cartilage erosion.²⁰ Also, document showed that cartilage-specific deletion of MMP-13 gene or the specific MMP-13 inhibitor, CL82198 could

Figure 5 The use of signaling pathway-specific inhibitors to investigate GRb1-mediated MMP-13, Notch1, JAG1, and CII expressions in cells (a) and cartilage (b). The former targets were significantly inhibited by signaling pathway-specific inhibitors compared with the IL-1β group and vehicle group ($P < 0.05$). In addition, increased expression of MMP-13 exhibited a greater reduction following treatment with GRb1 than by signaling pathway-specific inhibitor ($P < 0.05$). Data *in vivo* were consistent with the *in vitro* findings. # $P < 0.05$, ## $P < 0.01$ represent the protein expression in IL-1β group and IL-1β + GRb1 group compared to IL-1β + DAPT group. * $P < 0.05$ represents protein expression in IL-1β + DAPT group compared to IL-1β + GRb1 group. This experiment was repeated at least three times

decelerate the OA-like phenotype in the surgically induced OA model. The results of the present study demonstrated that the level of MMP-13 was up-regulated in IL-1β-induced SW 1353 cells and in rat osteoarthritic cartilages, while the level of CII was down-regulated. This result is consistent with studies above-mentioned.

Notch signaling plays a crucial role during embryogenesis, and studies have demonstrated that Notch is expressed in the pathological state of adulthood, like OA. Studies showed that Notch1 and the gliand, JAG1, were over-expressed in pathological cartilage.²¹ The study²² by Sassi *et al.* demonstrated that osteoarthritic chondrocytes

Figure 5 Continued.

induced decreases of CII and aggrecan and resulted in increases of MMP-13. Furthermore, the expressions of CII and aggrecan significantly increased in osteoarthritic chondrocytes treated with a Notch inhibitor DAPT, while the levels of MMP-13 were down-regulated.

GRb1, one of the most extensively studied of the ginsenosides, has a variety of biological activities, including anti-inflammatory and antiallergic activities.²³ In our previous study, we found that GRb1 at the concentrations of 20, 40, and 80 μ M did not have adverse effect on cell viability and could inhibit NO production and iNOS expression in IL-1 β -induced SW 1353 cells in a dose-dependent manner through suppressing NF-kappaB activation. In the current study, therefore, GRb1 at the concentration of 80 μ M was

used as the optimal concentration. It was documented that expressions of select Notch pathway genes, including Notch2 and Notch signaling, were regulated by IL-1 β and TNF- α .²⁴ Correspondingly, IL-1 β was applied to chondrocytes to mimic the microenvironment of OA for *in vitro* studies.²⁵ In the current study, IL-1 β was used to establish a cellular OA model in SW1353 cells, and it significantly promoted MMP-13 expression. The chondroprotective effects of GRb1 were evaluated on IL-1 β -induced SW 1353 cells with the Notch inhibitor DAPT treatment and GRb1 treatment. Conversely, GRb1 treatment was shown to be effective in protecting chondrocytes *in vitro* by down-regulating over-expression of MMP-13. Furthermore, the results *in vivo* were replicated *in vitro*. Experimental OA

was induced by ACLT in rats. The expressions of CII, MMP-13, Notch1, and JAG1 were detected by western blot and immunohistochemistry. The results showed that the decrease of CII was enhanced, and the increases of MMP-13, Notch1, and JAG1 were, respectively, suppressed with treatments of DAPT and GRb1. Moreover, the chondroprotective effects of GRb1 were superior to the Notch signaling inhibitor, DAPT. It may be related to the multiple properties of GRb1 and the multitarget mechanisms of GRb1 in OA.

Over the past decades, some MMP inhibitors have been failed to be studied as the candidates for the treatment of arthritis due to a few reasons, including non-specificity, toxicity, etc. There are also no MMP inhibitors that have been applied in clinical practice.^{26,27} Our work shows that GRb1 possesses potential anti-inflammatory property and inhibition of MMP-13 expression in OA, possibly through suppressing Notch signaling pathway to exert pharmacological effects.

Authors' contributions: WW and RHL conceived and designed the study; WW, LZ, and SZ performed laboratory experiments; WW, ZMW, XFR, and RHL analyzed data and interpreted the results; WW created the figures and drafted the manuscript; WW, LZ, ZMW, and RHL edited and revised manuscript; all authors approved the final version of the manuscript.

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