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ORIGINAL ARTICLE

Translational potential of ginsenoside Rb1 in managing progression of osteoarthritis

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Summary *Background:* Osteoarthritis (OA) is the most common degenerative joint disorder. Inflammatory cytokine plays an important role in OA progression. Previous studies have demonstrated that ginsenoside Rb1 would prevent inflammation and apoptosis in chondrocytes. However, we have not found any animal study reporting that Rb1 attenuates the severity of OA.
Objective: In this study, we used a rat anterior cruciate ligament transaction plus medial meniscus resection (ACLT + MMx) model of OA and a cell model, to investigate whether administration of ginsenoside Rb1 may attenuate the progression of arthritis.
Methods: In this *in vivo* study, 16-week-old male Sprague—Dawley rats were divided into three groups: Group 1 (sham control group), Group 2 (Rb1-treated group), and Group 3 (OA group). In Groups 2 and 3, OA was induced in the right knee joint with ACLT + MMx in rats. Then Group 2 received continuous infusion of ginsenoside Rb1 via osmotic mini-pumps implanted subcutaneously. At 4 weeks after treatment, the rats were sacrificed. Interleukin-1β (IL-1β) level was evaluated by enzyme-linked immunosorbent assay (ELISA); cartilage damage was assessed via histology (Safranin-O/fast green stain) and immunohistochemistry [matrix metalloproteinase-13 (MMP13) and type X collagen (Col X)]. For cell study, C5.18 (rat chondrocyte cell line) was used in this research. The effect of Rb1 on IL-1β-induced MMP13 or Col X

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expression level in C5.18 cells was investigated.
Results: In this *in vivo* study, characteristics of OA were present in the OA group, in contrast to less severe damage generally observed in the Rb1 treatment group: first, IL-1β level was significantly decreased, and second, cartilage degeneration was attenuated, as indicated by lower histologic damage scores and lower percentages of MMP13 or Col X-positive chondrocytes. In the cell study, the results showed that Rb1 treatment would relieve the MMP13 or Col X expression in C5.18 cells induced by IL-1β.
Conclusion: In the present study, we demonstrated that Rb1 can attenuate the progression or severity of arthritis by reducing inflammation.

Introduction

Osteoarthritis (OA) is one of the most common progressive joint disorders affecting our elderly population. The condition is pathologically characterized by cartilage degeneration, changes in subchondral bone matrix, and secondary synovial inflammation. Clinical presentation comprises pain, joint stiffness, and declining activity of daily functions [1—3]. Inflammatory cytokine plays an important role in OA progression [4—8]. For example, studies have shown that these inflammatory cytokines, including interleukin-1β (IL-1β) and stromal cell-derived-factor 1, are involved in lubricin catabolism and cartilage degeneration. Their mechanism resides in the upregulation of matrix metalloproteinase-13 (MMP13) that has been shown to cleave type II collagen and proteoglycans [5—8]. In addition, they can further inhibit the synthesis of the main constituents of the extracellular matrix, type II collagen, and aggrecan. These factors lead to the disruption of the normal articular cartilage homeostasis and the eventual breakdown of cartilage in OA.

Current research and treatment focus on identifying a method to regulate this mechanism, and thus, prevent or delay the breakdown of the articular cartilage or regenerate it. A class of natural products such as steroid glycosides and triterpene saponins, known as ginsenosides, has been identified as the principal active components in pharmacological studies of ginseng [9]. At the present time, over 40 ginsenosides have been identified, which show different biological activities based on structural differences. The most abundant ginsenoside Rb1 has been shown to have a variety of biological activities, including anti-inflammatory, antiapoptotic, and neuroprotective activities [10,11]. Previous *in vitro* studies have demonstrated that Rb1 would prevent IL-1β- or hydrogen peroxide (H_2O_2)-induced inflammation and apoptosis in chondrocytes [12,13]. In an *in vivo* study, Endale et al [14] proved that Rb1 would attenuate the severity of mouse collagen-induced arthritis. However, the effect of ginsenosides in a degenerative animal model remains unexplored.

The objective of this study is to investigate whether systemic administration of ginsenoside Rb1 may attenuate OA progression in a severe post-traumatic rat arthritis model.

Materials and methods

Animal surgery

All experiments were approved by the Animal Research Ethics Committee of the Chinese University of Hong Kong. In this study, 16-week-old male Sprague—Dawley rats, weighing 450—500 g, were used. The animals were allocated randomly into three groups. Group 1 involved an open arthrotomy and sutured closure, this served as the sham control ($n = 5$). In Groups 2 and 3, the rats were subjected to the anterior cruciate ligament transaction plus medial meniscus resection (ACLT + MMx) as described previously [15]. In brief, each rat was anaesthetized with a solution of 0.2% (vol/vol) xylazine and 1% (vol/vol) ketamine in phosphate-buffered saline (PBS), and after being shaved and disinfected, the right knee joint was exposed through a medial parapatellar arthrotomy approach. The patella was dislocated laterally and the knee was placed in full flexion, followed by anterior cruciate ligament (ACL) and medial cruciate ligament (MCL) transection with microscissors and resection of the medial meniscus. Group 2 is the Rb1-treated group; rats of this group received continuous infusion of ginsenoside Rb1 (PI & PI Technology Inc., Guangzhou, China) via osmotic mini-pumps ($n = 5$); The osmotic mini-pumps (model 2006; Alza Corporation, Mountain View, CA, USA) were inserted into small subcutaneous pockets over the dorsolateral thorax, created by blunt dissection after a small incision (~1 cm). Prior to insertion, 200-μL pump reservoirs were filled with Rb1 (300μM) dissolved in pure ethanol. The average pumping rate of the mini-osmotic pumps is 0.15 μL/h. Group 3 rats were left untreated and used as the OA controls ($n = 5$). In accordance with our animal ethics protocol, all the animal surgical procedures were performed under general anaesthesia and analgesic medications. After 4 weeks of treatment, all animals were euthanized, and then the right knees of animals were aspirated for analysis.

Blood collection and serum analysis

Blood (5 mL) was collected by cardiac puncture immediately after the animals were killed. The blood was centrifuged at 1800*g* for 10 minutes, and the separated serum

samples were then stored at −80°C until analysis. The level of IL-1β in the serum was measured using the IL-1β enzyme-linked immunosorbent assay (ELISA) kit (IL-1β ELISA kit, lot: EK0393; Boster, Pleasanton, CA, USA).

Histology and immunohistochemistry

After disarticulation of the right joint, both femur and tibia were dissected free of muscle and fixed in 10% formalin for 48 hours. Tibiae were then decalcified in 10% EDTA solution for 21 days and embedded in paraffin. Serial 5-μm-thick sagittal-oriented sections of the medial compartment of the knee joint were cut at intervals of 0 μm, 100 μm, and 200 μm, and collected on glass slides [16]. The sections were stained with Safranin-O/fast green. Cartilage degradation was quantified by Osteoarthritis Research Society International scores [17]. Three independent and blinded observers scored each section. Immunostaining was performed using a standard protocol. We incubated sections with primary antibodies to rabbit MMP13 (1:50, ab3208; Abcam, Cambridge, MA, USA) and collagen X (1:80, ab58632; Abcam, Cambridge, MA, USA) overnight at 4°C. For immunohistochemical staining, a horse radish peroxidase—streptavidin detection system (Dako, Chai Wan, Hong Kong) was subsequently used to detect the immunoactivity, followed by counterstaining with hematoxylin (Sigma—Aldrich, Poole, UK). Photographs of the selected areas were taken by a light microscope (Leica DMRB; Leica Cambridge Ltd, Cambridge, UK). We counted the number of positively stained cells in the whole tibia cartilage or subchondral bone area per specimen in three sequential sections (0 μm, 100 μm, and 200 μm) per rat in each group.

Cell experiment

C5.18 (rat chondrocyte cell line) was kindly gifted by Dr Tong Weixue. These cells were maintained in complete alpha-minimum essential medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum and 1% penicillin—streptomycin—neomycin (complete culture medium; all from Invitrogen Corporation, Carlsbad, CA, USA) in a 5% CO_2-humidified incubator at 37°C.

For induction by MMP13 or type X collagen (Col X), cells were transferred to serum-free DMEM (Dulbecco's Modified Eagle's Medium, Invitrogen Corporation, Carlsbad, CA, USA) and then treated with 10 ng/mL IL-1β (R&D Systems, Minneapolis, MN, USA) and Rb1 at 100 μg/mL. In the IL-1β group, cells were treated with 10 ng/mL IL-1β alone. In the control group, they were untreated except for a change in the medium. Cells were harvested after incubation for 24 hours.

RNA extraction and quantitative real-time polymerase chain reaction

Gene expression levels of MMP13 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of C5.18 cells induced by IL-1β were determined using real-time polymerase chain reaction (RT-PCR). Total cellular RNA was isolated with RNA Mini Kit (Invitrogen Corporation) and then reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Invitrogen Corporation) according to the manufacturer's instructions. RT-PCR was performed using the Step One Plus Real-Time PCR System (Applied Biosystems, Hong Kong) according to the manufacturer's instructions. The reaction conditions consisted of 15 μL reaction volumes. The relative quantification of gene expression was analysed with 2-ΔΔCT method and normalized with GAPDH expression level. The primers used are as follows: MMP13: forward (5′—3′) TCCTGATGTGGGTGAATACAATG, reverse (5′—3′) GCCATCGTGAAGTCTGGTAAAAT; GAPDH: forward (5′—3′) GCATGGCCTTCCGTGTTC, reverse (5′—′3′) GATGTCATCAT ACTTGGCAGGTTT.

Immunofluorescent assay

Col X expression levels of C5.18 cells induced by IL-1β were determined using an immunofluorescent stain. After induction by IL-1β, the cells were placed in 4% paraformaldehyde for 10 minutes and washed in phosphate-buffered saline twice. Immunofluorescent staining was performed using a standard protocol. We incubated the cells with primary antibodies to rabbit collagen X (1:100, ab58632; Abcam) overnight at 4°C. Secondary antibodies conjugated with fluorescence goat antirabbit CY3 (1:800; Life Technologies, Warrington, UK) were added, and cells were incubated at room temperature for 1 hour while avoiding light, followed by counterstaining of cell nuclei with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (Life Technologies, Warrington, UK). Photographs of the selected areas were taken by a microscope (Leica DMRB; Leica Cambridge Ltd, Cambridge, UK).

Statistical analysis

All statistical analyses were performed using the statistical software SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). The data were analysed using one-way analysis of variance (ANOVA). Assumptions of the analysis were assessed by the Shapiro—Wilk test of normality and Levene's test for homogeneity of variance. The result of Levnene's test was used to determine the *post hoc* testing strategy. If not significant, least significant difference test (LSD-t) *post hoc* test was employed. If Levnene's test was significant, the analysis of variance was followed by Dunnett's T3 *post hoc* test for unequal variance. Values of $p < 0.05$ were considered significant. Data were reported as mean ± standard deviation. The graphs were generated using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

Results

Ginsenoside Rb1 downregulates the level of IL-1β in serum of rat ACLT + MMx OA models

ELISA result of IL-1β showed that the IL-1β level in the Rb1-treated group (71.05 ± 10.49 pg/mL, $n = 5$, Figure 1) was reduced significantly compared with that in the OA group (118.69 ± 17.58 pg/mL, $n = 5$, $p < 0.05$, Figure 1). IL-1β

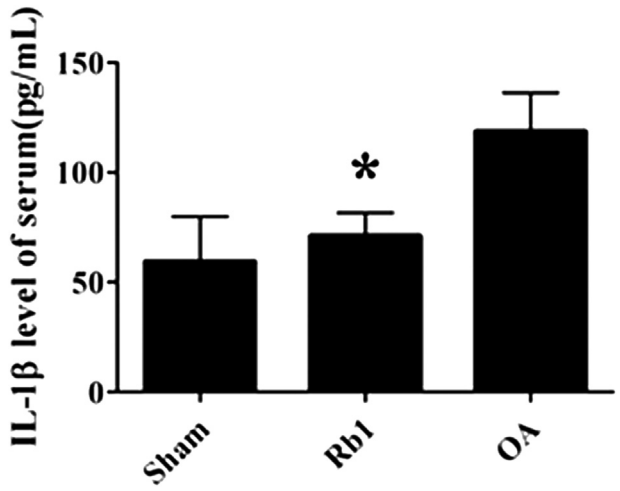

Figure 1 ELISA assay showing the IL-1β level in serum of the three groups, 4 weeks after surgery. It showed that ginsenoside Rb1 significantly downregulated the level of IL-1β, compared with the OA group ($n = 5$ per group). *$p < 0.05$. ELISA = enzyme-linked immunosorbent assay; IL = interleukin; OA = osteoarthritis.

level in the sham control group was the lowest (59.31 ± 20.51 pg/mL, $n = 5$, Figure 1). This result indicated that Rb1 treatment would relieve secondary inflammation in OA progression.

Ginsenoside Rb1 attenuates cartilage degeneration

Safranin-O staining revealed serious OA lesions in the cartilage in the OA group, with fewer OA changes being observed in the Rb1-treated group (Figure 2A). The Osteoarthritis Research Society International scores in the OA group reflected severe damage (14.86 ± 1.03, $n = 5$), whereas cartilage damage in the Rb1-treated group was significantly less (9.38 ± 1.89, $n = 5$, $p < 0.05$; Figure 2B), compared with the OA group. The Osteoarthritis Research Society International scores in the sham control group was the lowest (1.5 ± 0.41, $n = 5$, Figure 2B).

Moreover, the percentages of MMP13+ chondrocytes were lower in the Rb1-treated group (43.14 ± 7.96, $n = 5$) than in the OA group (62.87 ± 5.45, $n = 5$, $p < 0.05$; Figures 3A and 3B). Likewise, the percentages of type X collagen-positive chondrocytes of the Rb1-treated group (46.95 ± 3.83, $n = 5$) were lower compared with those of the OA group (64.6 ± 2.48, $n = 5$, $p < 0.05$; Figures 3A and 3C). In addition, the percentages of MMP13+ or Col X+ chondrocytes in the sham control group were the lowest (24.74 ± 3.51, $n = 5$ or 27.26 ± 2.53, $n = 5$, respectively; Figures 3A–C).

These results indicate that Rb1 would attenuate cartilage degeneration in OA development.

Ginsenoside Rb1 inhibits MMP13 or Col X expression in C5.18 cells induced by IL-1β

In the cell experiment, RT-PCR result showed that the relative MMP13 mRNA level in the Rb1 group (6.28 ± 1.45, $n = 5$, Figure 4) was reduced significantly compared with that in the IL-1β group (20.11 ± 5.89, $n = 5$, $p < 0.05$, Figure 4). In addition, MMP13 level in the control group was the lowest (0.9536 ± 0.04, $n = 5$, Figure 4). Immunofluorescent stain revealed that the number of Col X-positive cells in the Rb1-treated group was fewer than that in the IL-1β group. In the control group, the number of the Col X-positive cells was the lowest (Figure 5).

These results indicated that Rb1 treatment would relieve the MMP13 or Col X expression in C5.18 cells induced by IL-1β.

Discussion

OA is a common and disabling disease, but the mechanisms that drive this disease are not fully understood. However, increasing evidence has suggested that inflammatory cytokine is a main regulator of OA progression [4–8]. It is widely accepted that increased interleukin-1 beta (IL-1β) level plays an important role in this process. For example, a high level of

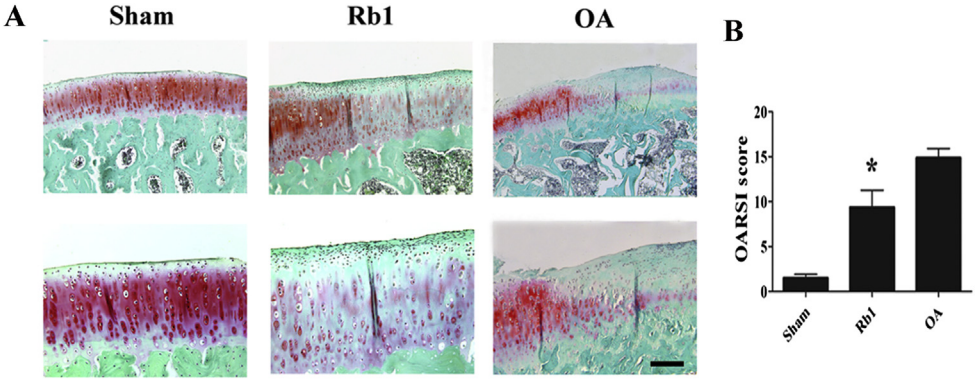

Figure 2 (A) Safranin-O and fast green staining of sagittal sections showing the subchondral tibia medial compartment. The articular cartilage was normal in the sham group. The Rb1-treated group showed fewer cartilage degenerative changes, whereas the OA group has the most severe degenerative changes. Scale bar, 400 μm (in top panels) and 200 μm (in bottom panels). (B) OARSI scores showed that cartilage degeneration in the OA group was most severe among the three groups, indicating that Rb1 treatment reduced the extent of cartilage damage; $n = 5$ per group. *$p < 0.05$, Rb1 group compared with the OA group. OA = osteoarthritis; OARSI = Osteoarthritis Research Society International.

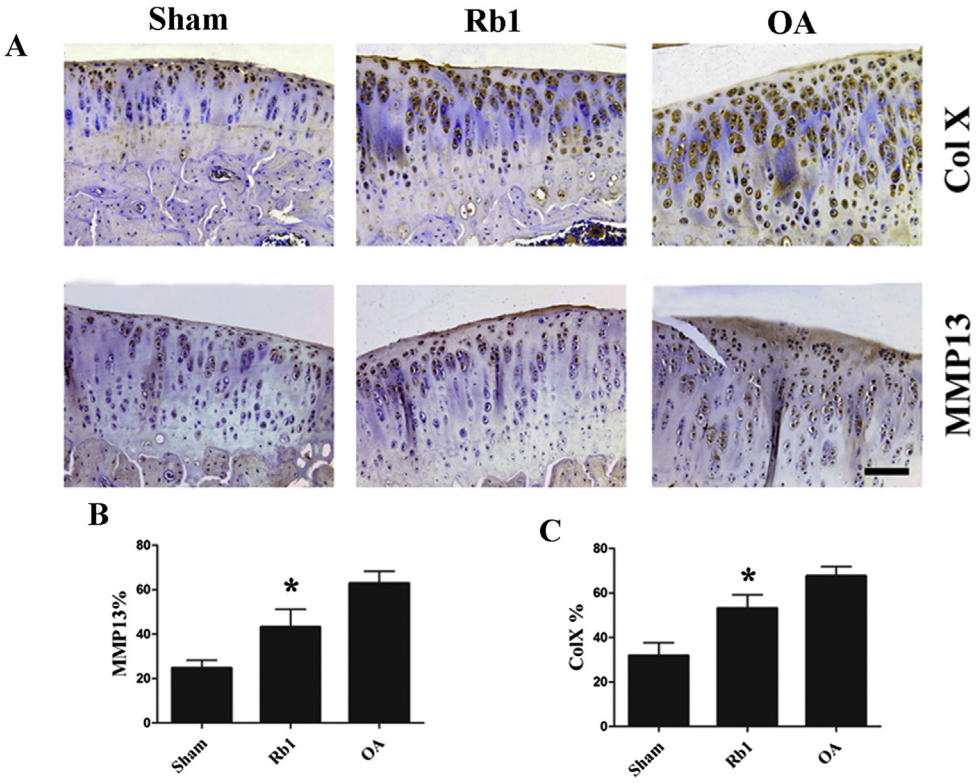

Figure 3 (A) Representative images showing the immunostaining results of COL X and MMP13 in the articular cartilage regions of the three groups; scale bar = 100 μm. (B) Semiquantitative analyses of the percentage of MMP13+ and COL X-positive chondrocytes (brown) in the articular cartilage of the three groups. Results showed that Rb1 treatment significantly reduced the number of MMP13+ and COL X+ chondrocytes in the articular cartilage compared with that of the OA group; $n = 5$ per group. *$p < 0.05$, Rb1 group compared with the OA group. Col X = type X collagen; MMP = matrix metalloproteinase; OA = osteoarthritis.

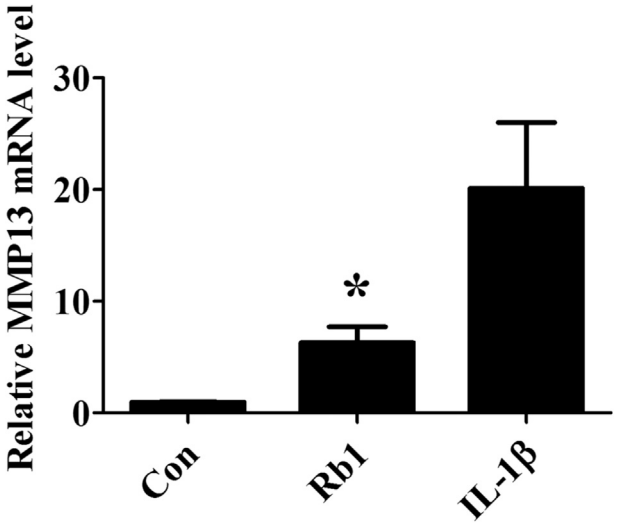

Figure 4 RT-PCR result showing the effect of Rb1 on IL-1β-induced MMP13 gene expression. The normalized gene expression levels are expressed as ratios of the copy number of the mRNA and that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA. Results showed that Rb1 significantly reduced the level of MMP13 gene expression in cells compared with that of the IL-1β-induced expression; $n = 5$ per group. *$p < 0.05$, Rb1 group compared with the IL-1β group. IL = interleukin; MMP = matrix metalloproteinase; RT-PCR = real-time polymerase chain reaction.

IL-1β would cause inflammation of the articular cartilage, stimulate the production of matrix metalloproteinases (MMPs), Col X, cyclo-oxygenase-2, and prostaglandin E2. These inflammatory cytokines can further inhibit the synthesis of the main constituents of the extracellular matrix, type II collagen, and aggrecan, and can disrupt the balance of metabolism in the articular cartilage [4,18,19]. Furthermore, it has been reported that apoptosis of chondrocytes can be induced by IL-1β [12], and chondrocyte apoptosis is thought to have a pivotal role in human and animal OA [20]. In our present research, we have used an ACLT + MMx OA model of rats and a cell model to study the benefits of ginsenoside Rb1 treatment for OA. We have demonstrated that the level of Rb1 has a positive effect of reducing the level of IL-1β. The cartilage damage score and the expression of MMP13 or COL X in chondrocytes are dramatically decreased in the Rb1 treatment group compared with those in the OA group. The results of the cell experiments showed that Rb1 can inhibit IL-1β-induced MMP13 or Col X expression in C5.18 cells. Taken together, these results indicated that the use of ginsenoside Rb1 might help chondrocytes regain their metabolic homeostasis.

Ginsenosides are unique saponins that exist only in ginseng, and they have a variety of pharmacological effects, including anti-inflammatory, antiapoptotic, and neuroprotective activities [10,11]. Ginsenoside Rb1 is most abundant among more than 40 ginsenosides. In fact, some researchers have demonstrated that ginsenoside Rb1 exerts

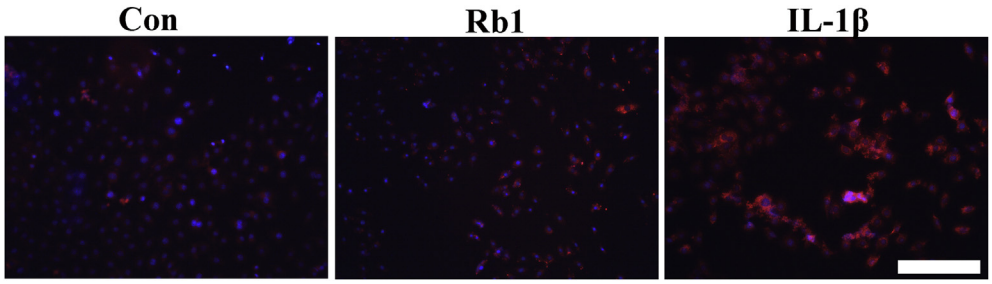

Figure 5 Immunofluorescent staining result showing the effect of Rb1 on IL-1β-induced Col X expression of C5.18 cells. This result indicated that the number of Col X-positive cells (red) in the Rb1 group was significantly reduced compared with the cells induced by IL-1β only. Scale bar, 400 μm. Col X = type X collagen; IL = interleukin.

some therapeutic effects on arthritis. In an *in vitro* study, Cheng et al [12], using a human articular chondrocyte model, found that Rb1 would prevent IL-1β-induced chondrocyte inflammation and apoptosis by decreasing the levels of prostaglandin E_2 (PGE_2), nitrogen dioxide ion (NO_2), MMP13, cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), and cysteine-aspartic acid protease-3 (caspase-3), and by increasing *Col2A1* gene expression levels. Another study showed that Rb1 has some anti-apoptotic effects on H_2O_2-treated chondrocytes by stabilizing the mitochondria and caspase-3 inhibition [13]. Lee et al [21] reported that Rb1 would inhibit MMP13 expression in IL-1β-treated chondrocytes. In an *in vivo* study, Endale et al [14] proved that in the mouse rheumatic arthritis model, Rb1 would attenuate the severity of arthritis by suppressing CD3+/CD69+, CD4+/CD25+, CD8+ T-cell, CD19+, B220/CD23+ B-cell, MHCII+/CD11c+, and Gr-1+/CD11b+ cell activations and anti-CII or anti-RF immunoglobulin G/immunoglobulin M, tumour necrosis factor-α, IL-1, IL-17, and IL-6 secretions, but stimulate IL-10 levels in the serum, joint, or splenocyte. Our present *in vivo* study is the first to report that Rb1 treatment would attenuate arthritis in an OA model. Our results are consistent with other similar research works mentioned above. We found that ginsenoside Rb1 shows the anti-inflammatory effect in this OA rat model; by downregulating the inflammation the severity of arthritis could be attenuated.

This study has some limitations: the number of animals per group was relatively low; in addition to IL-1β, other OA markers including MMP13 and MMP9 should also be used; the level of MMP13 or Col X should be tested in the rat chondrocytes by either RT-PCR or Western blot analysis. Future research should consider using a large number of animals in each group and a design to test multiple panels of OA markers at multiple time points.

In conclusion, we have studied the benefits of ginsenoside Rb1 treatment in a rat OA model and a cell model. We demonstrated that Rb1 can attenuate the severity of arthritis by reducing inflammation. These results imply that ginsenoside Rb1 may be a potentially useful drug for the treatment of OA patients.

Conflicts of interest

The authors have no conflicts of interest to declare.

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