Berberine induces dedifferentiation by actin cytoskeleton reorganization via phosphoinositide 3-kinase/Akt and p38 kinase pathways in rabbit articular chondrocytes

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Abstract

Osteoarthritis is a nonrheumatologic joint disease characterized by progressive degeneration of the cartilage extracellular matrix. Berberine (BBR) is an isoquinoline alkaloid used in traditional Chinese medicine, the majority of which is extracted from Huang Lian (*Coptis chinensis*). Although numerous studies have revealed the anticancer activity of BBR, its effects on normal cells, such as chondrocytes, and the molecular mechanisms underlying its actions remain elusive. Therefore, we examined the effects of BBR on rabbit articular chondrocytes, and the underlying molecular mechanisms, focusing on actin cytoskeletal reorganization. BBR induced dedifferentiation by inhibiting activation of phosphoinositide-3(PI3)-kinase/Akt and p38 kinase. Furthermore, inhibition of p38 kinase and PI3-kinase/Akt with SB203580 and LY294002, respectively, accelerated the BBR-induced dedifferentiation. BBR also caused actin cytoskeletal architecture reorganization and, therefore, we investigated if these effects were involved in the dedifferentiation. Disruption of the actin cytoskeleton by cytochalasin D reversed the BBR-induced dedifferentiation by activating PI3-kinase/Akt and p38 kinase. In contrast, the induction of actin filament aggregation by jasplakinolide accelerated the BBR-induced dedifferentiation via PI3-kinase/Akt inhibition and p38 kinase activation. Taken together, these data suggest that BBR strongly induces dedifferentiation, and actin cytoskeletal reorganization is a crucial requirement for this effect. Furthermore, the dedifferentiation activity of BBR appears to be mediated via PI3-kinase/Akt and p38 kinase pathways in rabbit articular chondrocytes.

Keywords: Berberine, chondrocytes, actin cytoskeleton reorganization, PI3-kinase/Akt, p38 kinase

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Introduction

Osteoarthritis (OA) involves the entire synovial joint, encompassing the cartilage, synovium, ligaments, tendons, menisci, and underlying bone.¹ OA is a common degenerative disease with high morbidity that is distinguished by degradation of the extracellular matrix, destruction of articular cartilage, and synovial inflammation. Furthermore, differentiated chondrocytes contain numerous cartilage-specific extracellular matrix molecules including type II collagen and proteoglycans.²

Berberine (BBR) is a botanical alkaloid present in the root and bark of a variety of plants, and it is used to treat intestinal infections in traditional Chinese medicine. It reportedly possesses various pharmacological activities such as anticancer, anti-inflammatory, and antioxidant.^{3–6} BBR

inhibits interleukin (IL)- β -induced proteoglycan release and nitric oxide (NO) production in IL-1 β -stimulated rat articular chondrocytes.⁷ BBR also prevented the release of collagen and proteoglycan from IL-1 β -treated rabbit cartilage and reduced matrix metalloproteinases (MMPs) in rabbit chondrocytes.⁸

Cartilage degradation is believed to result from the homeostatic imbalance between matrix anabolism and catabolism. In chondrocytes, actin filaments have a cortical distribution, along with the expression of type II collagen and aggrecans.⁹ Previous reports indicate that the actin cytoskeletal architecture is important for regulating the chondrocytes phenotype.^{10,11} Chondrocytes dedifferentiated by treatment with retinoic acid (RA)^{10,12} or serial monolayer culturing,¹³ exhibit changes in the actin

cytoskeletal architecture. Re-differentiation of chondrocytes with dihydrocytochalasin B is accompanied by disruption of the actin cytoskeleton.¹¹ In addition, the actin cytoskeleton regulates apoptosis in various cell types either positively or negatively, depending on the experimental system.^{14–16} A previous study by Kim *et al.*¹⁷ reported that alteration of the actin cytoskeleton by cytochalasin D (CD) regulates NO-induced apoptosis, dedifferentiation, and cyclooxygenase-2 (COX-2) expression via mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways.

However, the molecular mechanism underlying the BBR-induced dedifferentiation mediated by actin cytoskeletal reorganization has not been elucidated.

MAPK pathways regulate a variety of cellular responses such as cell growth, differentiation, inflammation, and apoptosis. In addition, the c-jun N-terminal kinase (JNK) and p38 pathways are activated by stress stimuli and inflammatory cytokines, whereas the extracellular signal-regulated kinase (ERK) pathway is stimulated mainly by a large variety of mitogens and growth factors.¹⁸ The phosphorylation of MAPK proteins emphasizes the importance of the balance between the phosphorylating kinases and dephosphorylating phosphatases in regulating these pathways. The balance between MAPKs and phosphatases contributes to determining the cell fate, including whether they undergo differentiation, proliferation, or apoptosis.¹⁹

Akt is phosphorylated by a dual phosphorylation mechanism involving a phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin (mTOR)-rictor complex.²⁰ Akt is a serine/threonine kinase that is an essential downstream molecule of phosphatidylinositol 3 (PI3)-kinase signaling and regulates multiple biological processes including cell survival, apoptosis, differentiation, and proliferation.²¹

We investigated the mechanisms of action of BBR in chondrocyte differentiation focusing on cytoskeletal architecture reorganization. Our findings suggest that the BBR-induced dedifferentiation of chondrocytes was mediated by cytoskeletal architecture reorganization via inhibition of PI3-kinase/Akt and p38 kinase pathways.

Materials and methods

Reagents and chemicals

BBR was obtained from Sigma-Aldrich (St. Louis, MO, USA) and prepared by dissolving in methanol to a final concentration of 0.05% methanol in the culture medium. Mouse anti-collagen type II monoclonal antibody (MAB8887; 0.2 µg/mL) was purchased from Chemicon International (Temecula, CA, USA). Mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (SC-166545; 0.2 µg/mL) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse anti-phospho-Akt polyclonal antibody (#9271; 0.2 µg/mL) and rabbit anti-phospho-p38 MAP kinase antibody (#9211; 0.2 µg/mL) were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-rabbit IgG antibody (A0545; 80 ng/mL) was obtained from Sigma-Aldrich and anti-goat IgG antibody (AP106P; 40 ng/mL) was

purchased from Chemicon International. Anti-mouse IgG antibody (ADI-SAB-100; 80 ng/mL) was obtained from Enzo Life Sciences International, Inc. (New York, NY, USA). All the other chemicals and reagents were of the highest grade commercially available.

Cell culture and experimental conditions

This study protocol was approved by the Ethics Committee of the Kongju National University. Primary rabbit articular chondrocytes were isolated from two-week-old New Zealand White Rabbits (Koatech, Pyeoungtaek, Republic of Korea). The cartilage was harvested from an area at a distance from the femur and calf. After washing with phosphate-buffered saline (PBS) supplemented with penicillin (100 unit/mL) and streptomycin (100 µg/mL), the cartilage was treated with 0.1% collagenase in TESCA buffer containing 50 mM N-Tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) and 0.36 mM calcium chloride, pH 7.4 at 37°C for 7 h. Isolated chondrocytes (2×10^4 cells/dish) were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (50 unit/mL), and streptomycin (50 µg/mL) in a 5% CO₂ incubator at 37°C. The medium was changed every two days after seeding and after three days, the chondrocytes were treated with either 50 µM or graded concentrations (0 µM, 10 µM, 30 µM, 50 µM) of BBR for predetermined times (0 min, 10 min, 30 min, 1 h, 3 h, 6 h, 12 h, 24 h) or 24 h, respectively. CD (Sigma-Aldrich, St. Louis, MO, USA) and jasplakinolide (JAS, Molecular Probes, OR, USA), which were used to induce reorganization of the actin cytoskeleton were added simultaneously with BBR. All data represent results of a typical experiment from at least four independent experiments.

Western blot analysis

Protein samples were extracted from the rabbit articular cartilage for western blot analysis. Briefly, chondrocytes were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride (NaCl), 1% Nonidet P-40, and 0.1% sodium dodecyl sulfate (SDS) supplemented with protease and phosphatase inhibitors. The mixture was incubated on ice for 30 min, centrifuged at 13,000 rpm for 10 min at 4°C, and then the supernatant was collected. Protein concentration was measured using a bicinchoninic acid assay, and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. After briefly washing in Tris-buffer saline Tween-20 (TBST), the membranes were blocked with 5% (w/v) nonfat dry milk in TBST at room temperature for 1 h. The membranes were incubated at 4°C with primary antibodies overnight followed by incubation with their respective secondary peroxidase-conjugated antibodies for 2 h. An enhanced chemiluminescence reagent was used to visualize the reactive bands, which were finally quantified using the LAS4000 (Fuji Film, Tokyo, Japan). The expression levels of target proteins were normalized to GAPDH.

Cell viability assay

Cells were seeded in a 96-well culture plates at a density of 1×10^4 cells/well in a final volume of 100 μ L DMEM and kept overnight for attachment. After incubation for three days, chondrocytes were treated with indicated concentrations (0 μ M, 10 μ M, 30 μ M, 50 μ M) of BBR for 24 h or 50 μ M BBR for varying times (0 h, 3 h, 6 h, 12 h, 24 h), and cells were incubated to grow for 24 h. After incubation, 10 μ L of MTT solution (Sigma-Aldrich) dissolved in the culture medium at the final concentration of 5 mg/mL was added to each well, and the plates were incubated for 4 h at 37°C. After completing the incubation, 100 μ L of solubilization buffer (10% SDS with 0.01 N HCl) was then added to solubilize MTT tetrazolium crystal, and the cells were incubated overnight at 37°C. Finally, the optical density was determined at 595 nm by using a microplate assay reader (Molecular Devices, Sunnyvale, CA, USA).

Reverse transcription-polymerase chain reaction

Total RNA samples were isolated using TRIzol (Life Technologies, Gaithersburg, MD, USA), and cDNA synthesis was performed with MaximeTM RT premix kit (iNtRON Biotechnology, Seongnam, Korea) in accordance with the manufacturer's protocol. The following primer sequences and polymerase chain reaction (PCR) conditions were used: type II collagen (370 bp product) 5'-GAC CCC ATG CAG TAC ATG CG-3' (sense) and 5'-AGC CGC CAT TGA TGG TCT CC-3' (antisense) with an annealing temperature of 52°C; sex determining region Y-box 9 (SOX-9, 386 bp product) 5'-GCG CGT GCA GCA CAA GAA GGA CCA CCC GGA TTA CAA GTAC-3' (sense) and 5'-CGA AGG TCT CGA TGT TGG AGA TGA CGT CGC TGC TCA GCT C-3' (antisense) with an annealing temperature of 60°C, and GAPDH (299 bp product) 5'-TCA CCA TCT TCC AGG AGC GA-3' (sense) and 5'-CAC AAT GCC GAA GTG GTC GT-3' (antisense) with an annealing temperature of 56°C. Reaction products were separated on an 0.8% agarose gel and stained with ethidium bromide.

Alcian blue staining

The cells (passage 0) used in this analysis were fixed in 3.5% paraformaldehyde in PBS for 15 min, and then stained with 0.1% Alcian blue in 0.1 M HCl overnight. The chondrocytes were washed three times with PBS buffer and 6 M guanidine HCl was added for 6 h. The production of sulfated proteoglycan was measured at 595 nm. Rabbit joint cartilage explants were fixed in 4% paraformaldehyde in PBS for 24 h at 4°C, washed with PBS, dehydrated with graded ethanol, embedded in paraffin, and sectioned at 4 mm thickness.²² Cartilage sections were deparaffinized in xylene and sequentially rehydrated in graded alcohol samples. After washing with PBS, cartilage sections were stained with 2.5% Alcian blue solution followed by three washes with 0.1 N HCl. After the sections were rinsed with PBS, the nuclei were counterstained with hematoxylin, and each section was observed under a light microscope (100 $\times$ magnification). The data represent results of a typical experiment from at least four independent experiments.

Immunofluorescence microscopy

Chondrocytes were plated on glass coverslips and treated with 50 μ M BBR for 24 h. Then, the cells were washed twice with PBS, fixed in 3.5% paraformaldehyde for 15 min at room temperature, washed thrice with PBS, and then permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. The fixed cells were subsequently washed with PBS and incubated for 2 h with antibody against type II collagen (MAB8887; 0.2 μ g/mL). Then, the cells were washed with PBS and incubated with secondary antibodies for 1 h at room temperature. F-actin was stained with rhodamine-phalloidin (Sigma), and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen, Burlington, ON, Canada). The cells were washed thrice with PBS and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Immunochemical staining

Immunohistochemistry was performed on the cartilaginous tissue to investigate the expression levels of type II collagen and sulfated proteoglycan. Articular cartilage specimens were fixed in 4% formaldehyde, embedded in paraffin, and cross-sectioned. For immunohistological analysis, sections were deparaffinized in xylene and sequentially rehydrated in graded alcohol samples. After washing with PBS, the sections were first incubated with 3% hydrogen peroxide for 15 min and then washed thrice with PBS. Then, the sections were immersed in 0.1 M sodium citrate buffer (pH 7.2) and heated at 90°C in a water bath for 40 min. Next, sections were blocked with 10% FBS for 10 min and incubated for 1 h at room temperature with a mouse anti-type II collagen antibody (4 μ g/mL) as the primary antibody. After washing with PBS, the primary antibody was detected using the avidin biotin conjugate method with a Dako kit (Dako Cytomation, Copenhagen, Denmark) according to the manufacturer's instructions. Peroxidase activity was detected using the Dako DAB kit (Dako Cytomation, Copenhagen, Denmark). Additional cartilage sections were stained with 2.5% Alcian blue solution followed by three washes with 0.1 N HCl. After the sections were rinsed with PBS, the nuclei were counterstained with hematoxylin, and each section was observed under a light microscope (100 $\times$ magnification).

Data analysis and statistics

Data are presented as the mean $\pm$ the standard error of the mean (SEM). The differences between each group were statistically analyzed using a one-way analysis of variance (ANOVA), and *P* values ≤ 0.05 were considered statistically significant.

Results

We tested the effect of BBR on cell viability of chondrocytes (Figure 1). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay indicated that BBR showed no significant toxicity at concentrations ranging of 10–50 μ M and, therefore, we used this range in this study (Figure 1).

Figure 1 BBR does not induce the toxicity in chondrocytes. Chondrocytes were treated with indicated concentrations (0 μ M, 10 μ M, 30 μ M, 50 μ M) of BBR for 24 h (a) or 50 μ M BBR for varying times (0 h, 3 h, 6 h, 12 h, 24 h) (b). Cell viability was determined by MTT assay. The effect of BBR on cell viability was expressed as percent. Cell viability compared with vehicle-treated control cells, which were assigned 100% viability. The data represent results of a typical experiment or mean values $\pm$ SEM from at least four independent experiments. * $p \leq 0.05$ vs. control cells. BBR: berberine

Treatment with BBR markedly altered the actin cytoskeletal architecture by lengthening the shape of the chondrocytes (long-shaped), as determined by the immunofluorescence staining (Figure 2). In addition, we examined the effect of BBR on dedifferentiation of chondrocytes, prior to determining the role of the actin cytoskeletal reorganization in BBR-induced dedifferentiation. We determine the effect of BBR on type II collagen expression by treating cells with 50 μ M or varying concentrations of BBR for the indicated times or 24 h, respectively (Figure 3).

The levels of type II collagen expression decreased after BBR treatment dose- and time- dependently, as determined by western blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) (Figure 3(a) and (b), respectively). BBR inhibited the differentiation of articular chondrocytes with accompanied loss of phenotype, as determined by the reduction in sulfated proteoglycan accumulation and type II collagen expression in chondrocytes or tissues with organ culture. Sulfated proteoglycan, the major component of cartilage, was stained with Alcian blue and treatment with BBR reduced its accumulation dose- and time-dependently in chondrocytes (Figure 4(a) and (b)). Chondrocytes treated with BBR showed a 70% decrease in sulfated proteoglycan accumulation, compared to the control chondrocytes (Figure 4(b)). As expected, BBR-treated cartilage explants exhibited a decrease in sulfated proteoglycan accumulation as well. Consistent with the result of the western blot analysis, BBR-treated cells revealed a loss of type II collagen, which was demonstrated by immunofluorescence staining (Figure 4(d)).

In the next experiments, we investigated the molecular mechanism of dedifferentiation by BBR. Treatment with BBR inactivated PI3-kinase/Akt and p38 kinase dose- and time-dependently, as detected by western blot analysis (Figure 5). Treatment with the PI3-kinase/Akt inhibitor, LY294002 (LY) or p38 kinase inhibitor, SB203580 (SB) enhanced the BBR-induced a loss of type II collagen, as

Figure 2 BBR induces actin cytoskeleton reorganization. Chondrocytes were treated with 50 μ M BBR for 24 h. Chondrocytes were stained for F-actin with rhodamine-conjugated phalloidin and analyzed using immunofluorescence microscopy. The data represent results of a typical experiment from at least four independent experiments. BBR: berberine; CON: control.

determined by western blot analysis and RT-PCR (upper and lower panels, respectively, Figure 6(a) and (b)). Consistent with the expression patterns of type II collagen, treatment with LY or SB enhanced BBR-reduced sulfated proteoglycan accumulation (Figure 6(c)). As expected, Alcian blue and immunohistochemical staining of cartilage explants indicated a decrease in sulfated proteoglycan accumulation and type II collagen expression (Figure 6(d)). These results suggest that the inhibition of the PI3-kinase/Akt and p38 kinase pathways is required for BBR-induced dedifferentiation of chondrocytes.

We previously showed that BBR-induced dedifferentiation via the PI3-kinase/Akt and p38 kinase pathways (Figures 3 and 4). The effects of actin cytoskeletal reorganization on BBR-induced dedifferentiation of chondrocytes were determined using immunofluorescence staining. Previous studies showed that treatment with CD, an inhibitor of actin polymerization, enhanced differentiation, whereas JAS, an inducer of actin polymerization, induced dedifferentiation in rabbit articular chondrocytes. The staining of actin cytoskeleton with phalloidin revealed that BBR counteracted the CD-induced inhibition of actin

Figure 3 BBR inhibits type II collagen expression. Chondrocytes were treated with indicated concentrations (0 μ M, 10 μ M, 30 μ M, 50 μ M) of BBR for 24 h (upper panel) or 50 μ M BBR for varying times (0 h, 3 h, 6 h, 12 h, 24 h; lower panel). (a) Expression of type II collagen and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected using western blot analysis. GAPDH was loading control. (b) Expression of type II collagen and GAPDH was detected using reverse transcription-polymerase chain reaction (RT-PCR). GAPDH was loading control. The data represent results of a typical experiment from at least four independent experiments. BBR: berberine

Figure 4 BBR causes dedifferentiation. Chondrocytes were treated with indicated concentrations (0 μ M, 10 μ M, 30 μ M, 50 μ M) of BBR for (a) 24 h or (b) 50 μ M for varying times (0 h, 3 h, 6 h, 12 h, 24 h). Accumulation of sulfated-proteoglycan was quantified by Alcian blue staining. (c) Cartilage was treated with 50 μ M BBR for 48 h. Synthesis of sulfated-proteoglycan was detected via Alcian blue staining. (d) Chondrocytes were treated with 50 μ M BBR for 24 h. Expression of type II collagen was determined using immunofluorescence staining. The data represent results of a typical experiment or mean values $\pm$ SEM from at least four independent experiments. * $p \leq 0.05$ vs. control cells. BBR: berberine; CON: control. (A color version of this figure is available in the online journal.)

polymerization but enhanced the JAS-induced actin polymerization (Figure 7(a)). In addition, treatment with BBR inhibited CD-induced type II collagen expression while it enhanced JAS-induced loss of type II collagen expression, as detected by immunofluorescence staining, western blot analysis, and RT-PCR (Figure 7(a) to (c)). Furthermore, consistent with the expression patterns of type II collagen, treatment with BBR inhibited CD-induced sulfated proteoglycan accumulation, whereas it enhanced JAS-induced decrease in sulfated proteoglycan accumulation (Figure 7(d)).

These results indicate that BBR-induced actin cytoskeletal reorganization regulates differentiation or dedifferentiation of chondrocytes. Accordingly, we examined whether cytoskeletal reorganization modulates BBR-induced dedifferentiation via the PI3-kinase/Akt and p38 kinase pathways. As depicted in Figure 7(b), treatment with CD in the presence of BBR increased PI3-kinase/Akt and p38 kinase activities, compared to the chondrocytes treated with BBR alone. In contrast to the effects of CD, treatment with JAS in the presence of BBR decreased the

PI3-kinase/Akt and p38 kinase activities, compared to the chondrocytes treated with BBR alone (Figure 7(b)).

Taken together, our finding indicates that BBR-induced dedifferentiation by actin cytoskeletal reorganization via the PI3-kinase/Akt and p38 kinase pathways in rabbit articular chondrocytes.

Discussion

Chondrocytes derived from articular cartilage are a unique cell type because their differentiated phenotype is reversible. The phenotype of chondrocytes is modulated by a balance of anabolic and catabolic factors that are required to maintain the homeostasis of cartilage tissue.¹ Differentiation of chondrocytes damages their phenotype and converts them to a fibroblastic morphology following exposure to various factors such as interleukin (IL)-1 β ,²³ RA,²⁴ endoplasmic reticulum (ER) stress,²⁵ and NO,²⁶ or during serial subculture²² *in vitro*. This disruption of cellular homeostasis is believed to be involved in the pathophysiology of arthritis.²⁷

Figure 5 BBR regulates phosphoinositide 3 (PI3)-kinase/Akt and p38 kinase pathways. Chondrocytes were treated with indicated concentrations (0 μ M, 10 μ M, 30 μ M, 50 μ M) of BBR for (a) 24 h or (b) 50 μ M for varying times (0 min, 10 min, 30 min; 1 h, 3 h, 6 h, 12 h, 24 h). Expression of type II collagen and GAPDH was detected using western blot analysis. GAPDH was loading control. The data represent results of a typical experiment from at least four independent experiments. BBR: berberine; GAPDH: glyceraldehyde 3-phosphate dehydrogenase

Figure 6 Dedifferentiation by berberine is regulated via phosphoinositide 3 (PI3)-kinase/Akt and p38 kinase pathways. (a)–(c) Chondrocytes were pretreated with 10 μ M SB203580 (SB, inhibitor of p38) or with 10 μ M LY294002 (LY, inhibitor of Akt) and then treated with 50 μ M BBR for 24 h. (a and b) Expression of type II collagen, pAkt, pp38, and GAPDH was detected using western blot analysis (upper panel). Expression of type II collagen and GAPDH was determined using reverse transcription-polymerase chain reaction (RT-PCR, lower panel). (c) Accumulation of sulfated-proteoglycan was quantified by Alcian blue staining. (d) Cartilage was pretreated with 10 μ M SB203580 (SB, inhibitor of p38) or with 10 μ M LY294002 (LY, inhibitor of Akt) for 6 h and then treated with 50 μ M BBR for 48 h. Synthesis of sulfated-proteoglycan was detected using Alcian blue staining. The data represent results of a typical experiment or mean values $\pm$ SEM from at least four independent experiments. * $p \leq 0.05$ vs. control cells. BBR: berberine; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; CON: control. (A color version of this figure is available in the online journal.)

BBR is an isoquinoline alkaloid that is present in a variety of plants of the genera *Berberis* and *Coptis*. These plants are commonly used in Chinese traditional medicine.²⁸ As one of the main active constituent of these plant extracts, BBR has demonstrated significant anti-inflammatory and immunosuppressive properties.²⁸ Previous studies have also determined that BBR inhibits withaferin-induced inflammation of chondrocytes.

However, there is no report on the effects of BBR on chondrocytes differentiation. Therefore, in this study, we demonstrated for the first time that BBR regulates dedifferentiation of rabbit articular chondrocytes dose- and time-dependently (Figure 3). To gain further insight into the mechanism underlying the capacity of BBR to inhibit

chondrocytes dedifferentiation, we investigated several intracellular signaling pathways. The MAPKs, including ERK, JNK and p38 kinase, are serine/threonine kinase pathways while the PI3-kinase/Akt pathway regulates various cellular processes including proliferation, apoptosis, survival, and differentiation.²⁹ Our previous study demonstrated the involvement of these signaling pathways in the regulation of dedifferentiation.³⁰ In this study, we determined the involvement of both MAPK and PI3-kinase; however, neither ERK-1/2 nor JNK was regulated in BBR-treated cells (data not shown).

We demonstrated that BBR regulates these functions in chondrocytes by regulating PI3-kinase/Akt and p38 kinase signaling. The actin reorganization by BBR prevented

Figure 7 Actin cytoskeleton reorganization by berberine causes dedifferentiation via phosphoinositide 3 (PI3)-kinase/Akt and p38 kinase pathways. (a)–(d) Chondrocytes or cartilage were treated with 1 μ M CD or 50 nM JAS with 50 μ M BBR for 24 h. (a) Chondrocytes were stained for F-actin with rhodamine-conjugated phalloidin and with type II collagen, and analyzed using immunofluorescence microscopy. (b) Expression of type II collagen, pAkt, pp38, and GAPDH was detected using western blot analysis. (c) Expression of type II collagen and GAPDH was determined using RT-PCR. (d) Accumulation of sulfated-proteoglycan was quantified using Alcian blue staining. The data represent results of a typical experiment or mean values $\pm$ SEM from at least four independent experiments. $^*p \leq 0.05$ vs. BBR-treated cells. BBR: berberine; CD: cytochalasin D; JAS: jasplakinolide; GAPDH: glyceraldehyde 3-phosphate dehydrogenase. (A color version of this figure is available in the online journal.)

activation of PI3-kinase/Akt and p38 kinase, which is likely responsible for chondrocytes dedifferentiation by BBR.

Interestingly, BBR-treated chondrocytes were alter morphology via actin cytoskeletal reorganization (Figure 2). This observation that actin cytoskeleton reorganization regulates chondrocytes phenotype is consistent with earlier reports.¹⁷ However, the molecular mechanisms mediating dedifferentiation in BBR-treated chondrocytes are yet to be elucidated. Consequently, we investigated the role of the actin cytoskeleton reorganization in BBR-treated chondrocytes.

CD is a fungal metabolite that prevents actin polymerization. It is suggested that actin depolymerization by CD causes differentiation.¹⁷ Furthermore, JAS is a macrocyclic peptide isolated from the marine sponge, which induces actin polymerization and enhances actin stability in a polymeric state, thereby inducing the dedifferentiation of chondrocyte.¹⁷ We further demonstrated the effects of BBR on dedifferentiation in the presence of CD or JAS. BBR inhibited CD-induced type II collagen, whereas it enhanced JAS-induced loss of type II collagen (Figure 6). Collectively, the results of our current investigation showed that actin cytoskeletal reorganization by BBR blocks differentiation and

induces dedifferentiation of chondrocytes. Therefore, BBR may be a potential candidate for further investigation for future use in the treatment or cartilage-related disorders including OA.

Author contributions: S-MY: Designed experiments, conducted research, and wrote manuscript; HC, G-HK, K-WC, and S-YS: Analyzed data; S-JK: Designed experiments, conducted research, analyzed data, and wrote manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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