Baicalin Promotes Chondrocyte Viability and the Synthesis of Extracellular Matrix through TGF- β /Smad3 Pathway in Mouse Chondrocytes

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Abstract

Background: Osteoarthritis (OA) is epidemic in the elderly people as a common chronic joint disease. By now, drug and surgical treatments are two main therapies for OA worldwide. Baicalin (BA) is a flavonoid monomer extracted from *Scutellaria baicalensis Georgi* and is reported that BA has anti-inflammatory, anti-deformation and anti-bacterial effects.

Methods: Micromass culture, alcian blue and Safran O (SO)/fast green staining were used to investigate chondrocyte viability and ECM synthesis in chondrocytes of all groups. The expression of SOX9 and Smad3 in chondrocytes of all groups were detected by western blot and RT-qPCR, the expression of aggrecan (AGG), type II collagen (Col2 α), MMP9/13 and ADAMTS5 were detected by RT-qPCR. In current study, we demonstrated that BA neutralized the down-regulation of chondrocyte viability and extracellular matrix (ECM) secretion, including AGG and Col2 α , induced by IL-1 β . As the key regulators of ECM, the down-regulation of SOX9, and the up-regulation of MMP9/13 and ADAMTS5 induced by IL-1 β were abolished by BA. Moreover, BA increased the nuclear translocation and phosphorylation of Smad3, one key mediator of TGF- β /Smads pathway. Interestingly, the addition of Smad3 inhibitor SIS3 reversed the promotions of BA on chondrocyte viability, ECM secretion, SOX9 expression, Smad3 nuclear translocation and Smad3 phosphorylation, and the down-regulation of BA on the expressions of MMP9/13 and ADAMTS5.

Conclusions: These results imply that BA can protect chondrocytes against IL-1 β -induced inflammatory injury through the acceleration of Smad3 phosphorylation and nuclear translocation in chondrocytes. This study demonstrates that BA may be a potential drug for OA clinical treatment.

Background

Osteoarthritis (OA) is epidemic in the elderly people as a common chronic joint disease [1]. OA has been characterized as joint pain, limb stiffness and others, as well as difficulty in movement which seriously affects the daily travel of patients [2]. The rapid growth of the epidemic brings a great burden on individuals and society [3]. By now, the treatments of OA in the world mainly include drug treatment and surgical treatment [4]. The surgical treatment with high cost is usually used in the advanced stage of OA patients [5], while the drug treatment of OA is trapped in the inhibition the inflammation. The drugs have an advantage in relieve inflammation and pain symptoms, but cannot prevent or delay the progress of OA completely [6]. So, it is urgent and necessary to seek new drugs for OA treatments.

Baicalin (BA) is a flavonoid monomer extracted from *Scutellaria baicalensis Georgi*. BA has anti-inflammatory, anti-bacterial and diuretic effects [7]. It has been reported that BA has a significantly inhibition on the inflammatory response and the expression of IL-1 β in chondrocytes [8]. IL-1 β is a pro-inflammatory factor and has shown to inhibit SOX9 expression and extracellular matrix (ECM) synthesis and to induce the productions of MMPs and ADAMTS5 [9]. BA can increase the transcription activity of SOX9, the synthesis of aggrecan (AGG) and type II collagen (Col2 α), two main components of cartilage

ECM [10] and inhibit the expressions of MMPs [11]. SOX9 is a key transcription factor in the development and differentiation of chondrocytes. The decreased expression of SOX9 leads to arrest differentiation of prechondrocytes and serious skeletal deformities [12]. MMPs are proteases and can degrade the ECM [13]. SOX9 is involved in several pathways including TGF- β /Smads pathway [14]. TGF- β /Smads pathway plays a key role in regulating SOX9 expression during cartilage formation [15]. Recent studies have shown that TGF- β /Smads could activate SOX9 expression [16]. As a key component of TGF- β /Smads pathway, Smad3 was confirmed to involve in chondrocyte differentiation during cartilage development [17]. TGF- β /Smads signaling pathway that results in accelerated chondrocyte maturation is inactivated by de phosphorylation of Smad3 [18]. Since BA can protect chondrocytes by up-regulating the expression of SOX9, but it remains unclear that whether BA protects chondrocytes through the activation of TGF- β /Smad3 by phosphorylating Smad3. Therefore, a chondrocyte inflammatory model *in vitro* is established by IL-1 β in this study. Cellular and molecular biology methods are used to investigate whether BA protects chondrocyte viability and ECM expression from inflammation through TGF- β /Smad3 pathway in mouse chondrocytes.

Materials And Methods

Reagents

BA has a chemical formula C₃₃H₄₀O₁₅ (Fig. 1a) and it was purchased from Shanghai PureOne Biotechnology Co., Ltd (#: 21967-41-9, Shanghai, China). BA was dissolved in dimethyl sulfoxide (DMSO) (Sigma Co, St Louis, Mo) and the solution (10⁻² mol/L) was stored at -20°C. The final concentration of DMSO in the culture was 0.01% (v/v). IL-1 β was purchased from R & D company (#: 201-LB-005, NASDAQ, USA). Smad3 inhibitor SIS3 was obtained from MedChemExpress Biological Technology Co., Ltd (#: CS-7115, Shanghai, China).

Cell vitality assay

Mouse chondrocyte (ADTC5) was a kind gift of Dr Yang from Basic Medical College of Jinan University. All cells were grown in DMEM medium (Gibco) containing 10% (v/v) FBS (Gibco), 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco) in a 5% CO₂ incubator at 37°C. The chondrocytes were passaged, resuspended and seeded in 96-well plates at a density of 4 × 10³ cells/well. Two hundred microliters of DMEM medium with different concentrations of BA (0, 10, 20 and 40 μ mol/L) or IL-1 β (0, 1, 2, 5 and 10 ng/mL) were added to the plates respectively next day. After culturing for 24 hours, cell viability was checked by MTS method (Promega Corporation, WI). The absorbance (OD value) at a wavelength of 490 nm was recorded using a microplate luminescence detector (Promega GloMax 96).

Micromass culture and Alcian blue & Safranin O staining

Twenty microliters of chondrocyte suspension (1×10^6 cells) was added to 12-well plates and incubated for 3 hours at 37°C for cell attachment. One milliliter of fresh DMEM medium containing various concentrations of drugs were added to each well. The experiments were divided into four groups, namely control group, IL-1 β group, IL-1 β + BA group and IL-1 β + BA + SIS3 group. Every three days, medium was changed. After 2 weeks, aggrecan (AGG) was checked by Alcian blue & Safranin O staining. The staining procedure was as below: wells were washed once with PBS (phosphate buffer saline) and then fixed for 20 min with 1.0 mL of 4% (v/v) paraformaldehyde (PFA). Subsequently, 0.5 ml of 1.0 % (w/v) Alcian blue (Sigma) (v/v) and 0.5 mL Safranin O solutions were added to each well and incubated for 20 min at room temperature. After 1 wash with 70% (v/v) ethanol and three washes with PBS, photomicrographs of the stained cell mass were obtained by a scanner (EPSON, V550, Japan).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Chondrocytes (2×10^5 /mL) were cultured in 6-well plates at 37°C in an incubator containing 5% CO₂. After 24 hours of culture, total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific). cDNA was synthesized with a PrimeScript™ Master Mix reagent kit (Takara Bio, Inc. Japan). qRT-PCR was performed with SYBR Premix ExTaq (Takara Bio, Inc.) using the qTOWER version 3.0 PCR system (Jena Industries, Inc.). The mRNA relative expressions of all target genes were analyzed using a formula ($2^{-\Delta\Delta T}$). GAPDH was used as the internal control. The primer sequences of all genes were shown in Table 1.

Table 1

Sequences of primers used for gene amplification.

Genes	Forward	Reverse
GAPDH	5'-ATTGTGCACCGCAAATGCTT-3'	5'-ACCACAGCACGATTGTGCGAT-3'
SOX9	5'-TCAACGGCTCCAGCAAGAACAAG-3'	5'-CTCCGCCTCCTCCACGAAGG-3'
Col2a	5'-GGTCCTCCTGGTCCTGGCATC-3'	5'-CGTGCTGTCTCAAGGTAAGTGTCTG-3'
AGG	5'-CGTTGCAGACCAGGAGCAAT-3'	5'-CTCGGTCATGAAAGTGGCGG-3'
MMP9	5'-AGAGACCACCACCACCACCAC-3'	5'-TGCCTGCCTCCACTCCTTCC-3'
MMP13	5'-CTACCATCCTGCGACTCTTGCG-3'	5'-CCACATCAGGCACTCCACATCTTG-3'
ADAMTS5	5'-AAGAGGAGGAGGAGGAGGAGGAG-3'	5'-AATGGTTGTGAGCTGCCGTATGG-3'

Immunohistochemistry

Chondrocytes ($1 \times 10^5/\text{mL}$) were seeded on the slides placed on 6-well plates. After 24 hours, the chondrocytes were fixed with PFA for 10 min at room temperature, and then were incubated with the primary antibody diluent of rabbit anti-p-Smad3 (# BM4033; 1:50; Boster) overnight at 4°C. The coverslips were covered with fluorescence secondary antibody (green, #A32723; 1:200; Thermo Fisher) against light for 30 min at room temperature. Mounting solution with DAPI (#C1005; Beyotime, China) was used to detect cell nuclei of chondrocytes. Fluorescence was detected by an inverted fluorescence microscope (CX31-32RFL; Olympus).

Western blot

The chondrocytes ($2 \times 10^5/\text{mL}$) were cultured in 6-well plates. The experimental groups were as follows: control group; IL-1 β (10 ng/mL) group; IL-1 β (10 ng/mL) + BA (20 $\mu\text{mol/L}$) group; IL-1 β (10 ng/mL) + BA (20 $\mu\text{mol/L}$) + SIS3 blocker (10 $\mu\text{mol/L}$). The extracted protein was loaded, separated and transferred onto a PVDF membrane by semi-dry transfer method. The membranes blocked by 5% Bovine Serum Albumin (# 11021029; Thermo Fisher), were soaked overnight at 4°C with solutions of primary anti-SOX9 (#5173; 1:1000; Cell Signal Technology), anti-p-Smad3 (# BM4033, 1:50, Boster), Smad3 (# BA4559; 1:50; Boster) and anti-GAPDH (#51745; 1:1000; Cell Signal Technology). The membrane was incubated with secondary antibody (# ARG65351; 1:3000; Arigo Biolaboratories) for 1 hour at room temperature next day. Images of the stained-protein bands were recorded using an ECL Western Blotting Substrate (#32106; Thermo Fisher) kit and quantified using Image lab system version 2.0. (Bio-Rad Laboratories, Inc.).

Statistical analysis

All data (n=3) were expressed as mean $\pm$ standard deviation and were calculated by SPSS 22.0 software. Student's t-test and One-way ANOVA were used for comparison between two groups and among many groups respectively.

Results

BA promotes the viability of ADTC5 chondrocytes

In order to confirm the optimal concentration of BA on chondrocytes viability, the chondrocytes were cultured with increasing concentrations of BA (0, 10, 20 and 40 $\mu\text{mol/L}$) for 24 hours. The chondrocytes viability was analyzed by MTS method. Various concentrations of BA (10-40 $\mu\text{mol/L}$) increased the cell viability ($p < 0.05$) (Fig. 1b). The most significant promoting effect on chondrocyte activity was found at 20 $\mu\text{mol/L}$ of BA. As shown in Fig. 1c, cell viability was significantly decreased in 10 ng/mL of IL-1 β group compared with control group ($p < 0.05$).

Combining MTS experiment results and literature [19], 20 $\mu\text{mol/L}$ of BA, 10 ng/mL of IL-1 β and 10 $\mu\text{mol/L}$ of SIS3 were used in subsequent experiments. The experimental groups were divided into 4 groups, namely control group (without any drugs), IL-1 β group, IL-1 β + BA group and IL-1 β + BA + SIS3 group. After 24 hours of culture, cell viability was analyzed by MTS method. As shown in Fig. 1d, the down-regulation of chondrocyte viability induced by IL-1 β was significantly abolished by BA treatment ($p < 0.05$). The addition of SIS3 significantly inhibited the protective effect of BA on chondrocyte viability.

BA promotes ECM synthesis in ADTC5 chondrocytes

Alcian blue and Safranin O staining are classic methods to detect ECM key component AGG [20]. As shown in Fig. 2a, IL-1 β treatment obviously down-regulated the expression of AGG in chondrocytes compared with control groups. On the contrary, the down-regulation of AGG induced by IL-1 β was obviously inhibited by BA. Moreover, the addition of SIS3 obviously reversed the protective effect of BA on the expression trend of AGG. The staining results indicated that BA promoted the secretion of AGG in chondrocytes and Smad3 inhibitor SIS3 disturbed the promotion of BA on the secretion of AGG.

By Alcian blue and Safranin O staining, we confirmed that BA promoted the expression of AGG in chondrocytes. Since AGG and Col2 α are two key components of ECM, we checked the expression of AGG and Col2 α in chondrocytes by qRT-PCR. Compared with control group, the expression of AGG and Col2 α in IL-1 β group was significantly down-regulated ($p < 0.05$, Fig. 2b & 2c). BA abolished the inhibition of IL-1 β on AGG and Col2 α expressions ($p < 0.05$). The addition of SIS3 reversed the inhibitory effect of BA on IL-1 β -induced down-regulation of AGG and Col2 α ($p < 0.05$). These results indicated that BA promoted ECM secretion in chondrocytes and Smad3 inhibitor SIS3 disturbed the promotion of BA on ECM secretion.

BA adjusts the expressions of ECM regulating genes in ADTC5 chondrocytes

As we know, SOX9, MMPs and ADMTS are key ECM regulators. SOX9 promotes ECM synthesis, and MMPs and ADMTS restrain ECM synthesis. By qRT-PCR and/or western blot, we checked their expressions in chondrocytes. Compared with control groups, the protein expression of SOX9 in IL-1 β group was significantly down-regulated ($p < 0.05$, Fig. 3a & 3b). BA abolished the inhibition of IL-1 β on protein expression of SOX9 ($p < 0.05$). The addition of SIS3 reversed the inhibitory effect of BA on IL-1 β -induced down-regulation in protein expression of SOX9 ($p < 0.05$). The change in mRNA expression of SOX9 was same as SOX9 protein (Fig. 3c). As shown in Fig. 3d-3f, the mRNA expressions of MMP9, MMP13 and ADAMTS5 in IL-1 β group were significantly up-regulated compared with control group, ($p < 0.05$). However, the inhibition of IL-1 β on the expressions of MMP9, MMP13 and ADAMTS5 was obviously abolished by BA treatment ($p < 0.05$). The addition of SIS3 reversed the protective effect of BA on chondrocytes treated by IL-1 β ($p < 0.05$). These results suggested that BA increased the expressions of

ECM synthesizing genes and decreased the expressions of ECM degrading genes and Smad3 inhibitor SIS3 disturbed the adjusting effect of BA on the ECM regulating genes in chondrocytes.

BA activates Smad3 in ADTC5 chondrocytes

Since Smad3 inhibitor SIS3 disturbed the promoting effect of BA on the secretion of ECM, it is reasonable to believe that BA may increase the activity of Smad3 which is related with Smad3 phosphorylation and nuclear localization and Smad3 activation may be one of the protective mechanisms of BA on chondrocyte viability and ECM synthesis. The effect of BA on the activation of Smad3 treated by IL-1 β was detected by immunofluorescence and western blot. As shown in Fig. 4a, Smad3 was expressed both in the cytoplasm and nucleus of chondrocytes in the control group, while Smad3 was mainly expressed in the cytoplasm of chondrocytes in the IL-1 β group. Immunofluorescence results showed that the Smad3 in the nuclear of chondrocytes in IL-1 β -induced groups was obviously less than that of BA + IL-1 β groups. Oppositely, the addition of SIS3 obviously reversed the nuclear translocation trend of Smad3. On the other side, as shown in Fig. 4b& 4c, the ratio of p-Smad3/Smad3 was down-regulated by IL-1 β treatment ($p < 0.05$). But BA treatment neutralized the decrease of p-Smad3/Smad3 induced by IL-1 β treatment ($p < 0.05$). Additionally, in the presence of IL-1 β and BA simultaneously, the addition of SIS3 obviously inhibited the protective effect of BA on chondrocytes. These results revealed that BA activates Smad3 in chondrocytes.

Discussion

OA is epidemic in the elderly people as a common chronic joint disease [1]. By now, drug and surgical treatments are two main therapies for OA worldwide [4]. Comparing with the high cost of surgical treatment, drug treatment of OA is more acceptable [5]. These drugs have an advantage in relieve inflammation and pain symptoms. It is reported that BA has anti-inflammatory, anti-deformation, anti-bacterial and diuretic effects [7, 8]. In current study, we demonstrate that BA increases the viability of chondrocytes and neutralized the inflammation induced by IL-1 β through TGF- β /Smad3 pathway.

IL-1 β is an inflammatory factor and is related to the progress of OA [20]. It has been shown that IL-1 β inhibits the secretion of Col2 α and AGG [21]. In addition, IL-1 β stimulates the releases of MMP9/13 and ADAMTS5 in chondrocytes which are responsible for the degradation of ECM [9, 22]. IL-1 β has been also shown to inhibit ECM synthesis through decreasing the expression of SOX9 [9]. By being treated with 10 ng/mL of IL-1 β , we successfully established the inflammatory and injury model of chondrocytes. After treated by IL-1 β , cell viability and the secretion of Col2 α and AGG were reduced; SOX9, one of ECM synthesis promoter was inhibited; on the contrast, MMP9, MMP13 and ADAMTS5, the ECM degradation promoters, were induced. However, BA significantly reversed the inhibition of IL-1 β on the secretion of Col2 α and AGG and the expression of SOX9 which mainly regulates the synthesis of Col2 α and AGG [23-25], and decreased the induction of IL-1 β on MMP9, MMP13 and ADAMTS5. The promoting effect of BA on the expression of SOX9 and ECM components Col2 α and AGG is consistent with previous report [26].

The transcriptional activations of SOX9 and TGF- β are necessary for prechondrocyte differentiation [27,28]. It is reported that TGF- β regulates the transcription of SOX9 in human chondrocytes via TGF- β /Smad2/3 signaling pathway [29]. It is suggested that the overexpression of Smad3 can significantly induce the formation of primary cartilage originated from human mesenchymal stem cell [23]. In addition, Smad3 enhances the transcription activity of SOX9 and Col2 α expression, while Smad3 silence inhibits the expression of SOX9 [30]. According to the literature [31], Smad2/3 is associated with SOX9 in TGF- β dependent manner and forms a transcription complex with SOX9 in the enhancer region of Col2 α . The TGF- β /Smad3 pathway plays a key role in SOX9-dependent cartilage formation through up-regulating Smad3 phosphorylation [32]. In this study, BA significantly inhibited the down-regulation of Smad3 phosphorylation and Smad3 cytoplasm transfer induced by IL-1 β . However, the effect of BA on Smad3 phosphorylation and nuclear translocation was blocked by Smad3 inhibitor SIS3. The mechanisms underlying the effect of BA on the phosphorylation and nuclear translocation of Smad3 needs further study to clarify.

Conclusions

Taken together, present study demonstrates that BA may increase cell viability and cartilage ECM synthesis such as Col2 α and AGG. BA increases the expression of SOX9 and inhibits the expression of MMP9, MMP13 and ADAMTS5 by increasing Smad3 phosphorylation and nuclear translocation. This study provides a theoretical basis for BA as a clinical candidate drug for OA treatment, the main results are illustrated in Fig. 5.

Abbreviations

BA: Baicalin; DMEM: Dulbecco's Modified Eagle Medium; ECM: extracellular matrix FBS: Fetal bovine serum; OA: Osteoarthritis; AGG: Aggrecan; Col2 α : Type II collagen; RT-qPCR: Quantitative reverse transcription polymerase chain reaction.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

PW, AL and ZL designed the study. PW, SZ and XX performed the most of experiments, SZ and XX managed cell culture, PW analyzed the data. PW, AL and ZL wrote the manuscript. AL and SZ contributed in the experimental design. All authors have read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the research ethics committee of Guangzhou Red Cross Hospital, Guangzhou, 510220, China, the approval number is 2019-010-01.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

BA promotes the viability of ADTC5 chondrocytes. a Structural formula of BA. b The cell viabilities of ADTC5 chondrocytes which were cultured with increasing concentrations of BA (0, 10, 20 and 40 μmol/L) for 24 hours were checked by MTS analysis. c The cell viabilities of ADTC5 chondrocytes which were cultured with increasing concentrations of IL-1β (1, 2, 5 and 10 ng/mL) for 24 hours were checked by MTS analysis. d The cell viabilities of ADTC5 chondrocytes which were cultured without or with IL-1β (10 ng/mL), BA (20 μmol/L) and SIS3 (10 μmol/L) for 24 hours were checked by MTS analysis

Figure 2

BA promotes ECM synthesis in ADTC5 chondrocytes. a The chondrocyte masses were stained by SO and Alcian blue staining. The relative expression of AGG (b) and Col2α (c) by qRT-PCR in chondrocytes which were cultured without or with IL-1β (10 ng/mL), BA (20 μmol/L) and SIS3(10 μmol/L) for 24 hours. *P < 0.05 compared with corresponding group.

Figure 3

BA adjusts the expressions of ECM regulating genes in ADTC5 chondrocytes. The protein expression of SOX9 (a) and the ratio of SOX9 against GAPDH (b) in chondrocytes which were cultured without or with IL-1β (10 ng/mL), BA (20 μmol/L) and SIS3 (10 μmol/L) for 24 hours. The relative mRNA expression of SOX9 (c), MMP9 (d), MMP13 (e) and ADMTS5 (f) in chondrocytes which were cultured without or with IL-1β (10 ng/mL), BA (20 μmol/L) and SIS3 (10 μmol/L) for 24 hours. *P < 0.05 compared with corresponding group.

Figure 4

BA activates Smad3 phosphorylation and nuclear localization in ADTC5 chondrocytes. (a) Smad3 localization in chondrocytes which were cultured without or with IL-1 β (10 ng/mL), BA (20 μ mol/L) and SIS3 (10 μ mol/L) for 24 hours checked by immunohistochemistry staining. Smad3 expression (b) and relative phosphorylation (c) checked by western blot in chondrocytes which were cultured without or with IL-1 β (10 ng/mL), BA (20 μ mol/L) and SIS3 (10 μ mol/L) for 24 hours. *P < 0.05 compared with corresponding group.

Figure 5

Schematic diagram of research ideas.