Anti-Inflammatory and Chondroprotective Effects of Platelet-Derived Growth Factor-BB on Osteoarthritis Rat Models

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Research Article

Keywords: platelet-derived growth factor, osteoarthritis, SOX-9, RunX-2

Posted Date: August 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-786857/v1>

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Abstract

Background: It has been proved that PDGF-BB could protect the chondrocytes via multiple mechanisms. The present study aims to further investigating the anti-inflammatory and chondroprotective effects of platelet-derived growth factor (PDGF)-BB on osteoarthritis (OA).

Methods: We established an osteoarthritis model in Sprague-Dawley rats through intraarticular injection of 3 mg/50 μ L monosodium iodoacetate (MIA). The effects of PDGF-BB on MIA-induced OA were assessed by histopathological staining, real-time PCR, and western blotting. Chondrocytes were transfected with or without SOX-9 siRNA, induced by MIA and treated with PDGF-BB, and cell viability, proinflammatory cytokines, and specific markers were analyzed. For mechanistic analysis, the expression of p-SOX-9/SOX-9, p-RunX-2/RunX-2, and p-PKA/PKA and activation of the JAK2/STAT3, PI3K/AKT, and p38 pathways were analyzed.

Results: PDGF-BB significantly inhibited the degeneration of cartilage in MIA-induced OA with reductions in proinflammatory cytokines (IL-1, IL-6, collagen α , MMPs and ADATMS) and increases in collagen II, aggrecan, and integrin α 5 β 1, and these effects could be reversed by a PDGFR- β antagonist. Consistently, the in vitro results showed similar effects. PDGF-BB treatment suppressed the activation of the JAK2/STAT3, PI3K/AKT, and p38 signaling pathways after MIA induction in both cartilage and chondrocytes. In addition, activation of PKA by PDGF-BB enhanced SOX-9 phosphorylation, which increased anabolic activity, and attenuated RunX-2 phosphorylation, which decreased catabolic activity. The PKA inhibitor and SOX-9 siRNA suppressed PDGF-BB-mediated chondroprotection.

Conclusions: PDGF-BB could attenuate OA development by regulating the JAK2/STAT3 PI3K/AKT and p38 signaling pathways by inhibiting inflammation and enhancing cell proliferation via PKA-mediated regulation of SOX-9/RunX-2.

Background

Osteoarthritis (OA) is a degenerative disease of the joints that is characterized by cartilage degradation, osteophyte formation, and synovial inflammation [1]. The occurrence and development of osteoarthritis are associated with multiple factors, such as age, joint load, and trauma [2]. Previous studies have shown that the pathogenesis of OA is due to degeneration, inflammation, necroptosis, and apoptosis [3]. However, the pathogenesis of OA is still not fully understood. In general, OA is an inflammatory disease characterized by the production of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF- α) and interleukin 1 (IL-1), which are upregulated, and the synthesis of cartilage-associated degradation substances, such as matrix metalloproteinases (MMPs) aggrecanase (ADAMTS), cyclooxygenase-2 (COX-2), and nitric oxide synthases (NOSs), which in turn cause continuous damage to cartilage [4–6].

Traditionally, except for surgery for severe cases, treatments for OA mainly focus on relieving pain and improving joint function. For example, nonsteroidal anti-inflammatory drugs (NSAIDs), IL-4, and IL-1 receptor antagonists have been approved to have obvious anti-inflammatory effects; however, these

treatments are not effective for cartilage repair and induce only symptomatic relief [7]. Chondrocytes are the only resident cells in articular cartilage and are involved in the pathological process of OA through degeneration, inflammation, and apoptosis, which suggests the necessity of treatments that protect chondrocytes. In addition to anti-inflammatory effects, attenuation of OA progression via chondroprotection without side effects has been given increasing attention. Therefore, it is still a challenge for clinicians to discover new components to cure OA by inhibiting inflammation and attenuating the degradation of chondrocytes rather than just relieving symptoms.

Platelet-derived growth factor (PDGF) was originally identified in platelets, and the PDGF family includes four homodimeric proteins (PDGF-AA, -BB, -CC, and -DD). In particular, PDGF-BB has gained prominence in a variety of orthopedic applications due to its capacity for tissue repair. A study conducted by Li-Jen Yuan et al. proved that PDGF-BB could increase the growth of rabbit chondrocytes via upregulated mRNA expression of PDGF receptor [8]. When cultured with human articular chondrocytes in vitro, recombinant PDGF-BB significantly increased the proliferation rate in chondrocytes compared to that of the control group [9]. Similarly, a subsequent study showed that PDGF-BB suppressed apoptosis by regulating NF- κ B and PI3K/AKT pathway in IL-1 β -induced human chondrocytes [10]. In 2019, an in vitro study used recombinant PDGF-BB and hyaluronic acid in combination as potent growth factors to stimulate the increased formation of chondrocytes [11]. Greene MA et al. noted that targeting signaling proteins, such as JAK1/2, was a better therapeutic approach to restoring the balance in anabolic and catabolic activity in cartilage than targeting PI3K/AKT signaling [12]. However, the application of PDGF-BB in OA and the potential mechanisms are still unclear and need to be further discussed. Therefore, as a potential new treatment to protect cartilage in the context of OA, it is critical to elucidate the molecular and cellular mechanisms involved in cartilage inflammation and chondrocyte responses to PDGF-BB.

To advance our understanding of the effects of PDGF-BB, we first established an OA model induced by MIA (sodium iodoacetate) and administered PDGF-BB treatment to investigate whether PDGF-BB could inhibit inflammation and exert chondroprotective effects. Second, we investigated how PDGF-BB treatment affected the degenerative processes of chondrocytes using various molecular techniques.

Methods

Agents and antibodies

PDGF-BB was purchased from Sigma-Aldrich (St. Louis, MO, USA). Monosodium iodoacetate, which was used to induce OA in rats, was purchased from Sigma-Aldrich (St. Louis, MO, USA). The PKA (PKAi, H 89 2HCl) and PDGF receptor β (PDGFRi, Ki20227) inhibitors were purchased from Selleck (Shanghai, China). Rabbit anti-PDGF, PDGFR, p-PKA/PKA, p-JAK2/JAK2, p-STAT3/STAT3, p-PI3K/PI3K, p-AKT/AKT, and p-p38/p38 primary antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-p-SOX-9/SOX-9 and p-RunX-2/RunX-2 primary antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were obtained from Proteintech (Chicago, IL, USA).

Cell culture

Primary chondrocytes were isolated from the knee joints of 1-week-old Sprague-Dawley rats by digestion with 0.25% trypsin for 30 min followed by digestion with 0.2% type II collagenase at 37°C for 8 h. Chondrocytes were collected in sterile culture bottles and cultured in DMEM/Ham's F-12 (1:1) culture medium containing 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin at 37°C with 5% CO₂.

When the chondrocytes had spread to approximately 80–90% of the culture vessel (approximately 2.5×10^6 /vessel), cell passage was performed. Thrice-passaged chondrocytes were seeded in six-well plates (10^5 cells per well) and harvested after 6 h for further experiments. Cell climbers were placed in the bottom of each plate for immunofluorescence staining.

Cell transfection

Recombinant adenovirus-mediated SOX-9 siRNA was synthesized by GeneChem (Shanghai, China). For cell transfection, chondrocytes were plated in 24-well plates and grown to 60% confluence, after which the cells were transfected with 10 pmol/50 nM siRNA against SOX-9 (Invitrogen). Transfection with 50 nM scrambled siRNA was used as a control. Transfection efficiency was monitored based on the quantification of transfected green fluorescence-labeled irrelevant siRNA by FACS analysis after 24 h of incubation.

Osteoarthritis-induced chondrocytes and treatments

To examine the effect of PDGF-BB on MIA-induced chondrocytes, the cells were divided into the control, OA, OA + PDGF-BB (100 ng/mL), and OA + PDGF-BB + PDGFRi (250 µM) groups. To examine the effect of SOX-9 on PDGF-BB-treated chondrocytes, normal chondrocytes were transfected with siRNA and divided into the control, OA, OA + PDGF-BB (100 ng/mL), OA + PDGF-BB + PDGFRi (250 µM), OA + PDGF-BB + PKAi (50 µM), and OA + PDGF-BB + SOX-9i groups. For each group, the chondrocytes were cultured in DMEM/F-12 at a density of 1×10^5 cells/mL in 6-well plates and pretreated with PDGF-BB (with or without other reagents) for 1 h prior to stimulation with 5 µM MIA for 24 h.

Animals

All experiments were approved by the Tongji Medical College Council and the Animal Care Committee of Huazhong University of Science and Technology (Wuhan, China). The study procedures were performed under the guidelines of the Care and Use of Laboratory Animals (Science and Technology Department of Hubei Province, China, 2005). Male Sprague-Dawley (160–200 g) rats were fed a normal diet and housed with a 12 h light/dark cycle under controlled humidity and temperature conditions (25°C). Animals were divided into five groups: the control group, OA group, PDGF-BB (100 ng/µL, 30 µL in total) group, PDGF-BB + PDGFRi (250 µM, 30 µL in total) group, and PDGF-BB + PKAi (50 µM, 30 µL in total) group.

Induction and assessment of the OA model

PDGF-BB was reconstituted in 0.9% sterile saline to achieve a concentration of 1 ng/mL. Bilateral knee joint OA was induced by intraarticular injection of 3 mg/50 µl monosodium iodoacetate under 1% pentobarbital anesthesia (80 mg/kg i.p.) using a 27-gauge needle inserted through the patellar tendon. X-ray detection was performed after 2 weeks to evaluate changes in the joint structure using a Faxitron MX-20 radiography system (Faxitron X-ray Corp, Wheeling, IL). Then, PDGF-BB (with or without PDGFRi and PKAi) injections were performed once per week for four weeks.

Cell viability assay and enzyme-linked immunosorbent assays (ELISAs)

Cell viability was determined using a CCK-8 kit according to the manufacturer's instructions. The absorbance at 450 nm was measured with a microplate reader (Leica Microsystems, Germany). ELISA was conducted using commercial kits (R&D Systems) according to the manufacturer's instructions. All experiments were performed in triplicate.

Histological analysis

After 4 weeks, the rat knee joints were extracted and fixed in 4% paraformaldehyde for 24 h and transferred to 10% EDTA until the tissue was decalcified. Then, the tissues were embedded in paraffin, and 4-µm-thick paraffin sections were prepared for histological staining. Hematoxylin and eosin staining was carried out to assess structural changes in the cartilage, and safranin-O fast green staining was used to measure cartilage degeneration. The osteophyte score was quantified as previously described [13]. Three observers who were blinded to the groups quantified OA severity using the Mankin scoring system[13], and the 3 scores were averaged.

Immunofluorescence analysis

The histology slides were prepared as described above. The cell climbing slides were washed three times with ice-cold PBS and fixed with 4% paraformaldehyde for 20 min. Antibodies were diluted according to the manufacturer's instructions. After deparaffinization and rehydration, the samples were incubated overnight with the diluted antibodies at 4°C. Fluorescein-labeled secondary antibodies were added to the samples, and incubation was continued for 1 h at room temperature. Nuclei were stained with DAPI, and the slides were scanned using an LSM 710 confocal microscope (Zeiss) with an EC-Plan-Neofluar 40×/1.3 oil immersion objective. Quantitative data were obtained from the fluorescence intensity measurements by using ZEN 2009 software (Zeiss).

Real-time polymerase chain reaction (RT-PCR)

A total RNA kit (Takara, Japan) was used to isolate RNA from rat knee joint cartilage according to the manufacturer's instructions. An RNA PCR kit (Takara, Japan) was used to conduct reverse transcription and cDNA synthesis. The real-time polymerase chain reaction was performed using a Step One SYBR Green Mix Kit (Takara, Japan) and an ABI Prism Sequence Detection System (Applied Biosystems, USA) according to the manufacturer's instructions. The PCR primers are shown in Table 1. All relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Table 1
Primer sequences of RT-PCR.

Genes	Sequence (5' -3')
collagen II	F: 5'- GTCCTACAATGTCAGGGCCA-3'
	R: 5'- ACCCCTCTCTCCCTTGTAC-3'
aggrecan	F: 5'-CCGCTGGTCTGATGGACACT-3'
	R: 5'-AGGTGTTGGGGTCTGTGCAA-3'
integrin α 5	F: 5'-GGCAGCTATGGCGTCCCACTGTGG-3
	R: 5'-GGCATCAGAGGTGGCTGGAGGCTT-3'
integrin β 1	F: 5'-GGACGCTGCGAAAAGATGAA-3'
	R: 5'-CACATCGTGCAGAAGTAGGC-3'
collagen X	F: 5'-TTCTGCTGCTAGTGTCCCTTGACG-3'
	R: 5'-GGGATGAAGTATTGTGTCCTGGG-3'
MMP-3	F: 5'-GTACCAACCTATTCCTGGTTGC-3'
	R: 5'-CCAGAGAGTTAGATTTGGTGGG-3'
MMP-13	F: 5'-CGTAGTGATCAGAGCCAAGC-3'
	R: 5'-TCTGCCTTTCCTGCAATTAGA-3'
ADAM-4	F: 5'-GGAATGGTGGAAAGTATTGTGAGG-3'
	R: 5'-GGTCGGTTCGGTGGTTGTAG-3'
ADAM-5	F: 5'-GGCCTTCCAAATAAATACATTCA-3'
	R: 5'-TTAGCUAGGGAUCGACAGAAA-3'
IL-6	F: 5'-AGAGGAGAC TTCACAGAGGA-3'
	R: 5'-CCAGTTTGGTAG CATCCATC-3'
IL-8	F: 5'-CTCCAGCCACACTCCAACAGA-3'
	R: 5'-CACCTAACACAAAACACGAT-3'
TNF- α	F: 5'-CCTACCTTCAGACCTTCCAGAT-3'
	R: 5'-GGCCTTCCAAATAAATACATTCA-3'
iNOS2	F: 5'-CATTGGAAGTGAAGCGTT TCG-3'
	R: 5'-CAGCTGGGCTGTACAAACCTT-3'
COX-2	F: 5'-TGTATGCTACCATCTGGC TTCGG-3'

Genes	Sequence (5' -3')
	R: 5'-GTTTGGAAACAGTCGCTCG TCATC-3'

Western blotting

Total proteins were extracted from knee joint cartilage, and protein concentrations were determined using a BCA protein assay kit (Pierce). Total proteins (40 g) were separated by 10% SDS-PAGE and then electrotransferred onto nitrocellulose membranes. After being blocked with TBST containing 5% skim milk, the membranes were incubated at 4°C overnight with primary anti-PDGF antibodies (1:200; Abcam). The membranes were incubated with secondary antibodies at 37°C for 2 h (goat-anti-rabbit). After coloration, immunoreactive bands were obtained. Quantitative data were obtained from the results of densitometric methods by using AlphaEaseFC software (Alpha Innotech).

Statistical analysis

Statistical analyses were performed using SPSS 21.0 software. The normality of distribution was tested by a QQ plot. The data were analyzed using repeated measures ANOVA. A post hoc test of the p-value was performed by a Bonferroni correction. A value of $p < 0.05$ was considered statistically significant.

Results

PDGF-BB enhances the proliferation of chondrocytes in vitro

To investigate the effect of PDGF-BB on MIA-induced chondrocytes, cultured third-generation chondrocytes were stimulated with or without MIA and then stimulated with PDGF-BB or PDGF-BB + PDGFRi. The CCK-8 assay results showed that PDGF-BB improved cell viability and enhanced proliferation after MIA stimulation (Fig. 1A). In addition, a PDGFRi antagonist reversed the proliferative effects of PDGF-BB on chondrocytes.

PDGF-BB relieves cartilage degeneration in MIA-induced osteoarthritis in vivo

Two weeks after MIA treatment, X-ray imaging confirmed that all treated rats successfully developed OA. To investigate the anti-osteoarthritic effect of PDGF-BB in vivo, we observed changes in cartilage by gross observation, X-ray imaging, and HE and toluidine blue staining. HE staining of cartilage in the OA model showed chondrocyte loss, thinning proteoglycan, and cartilage necrosis compared to those in normal cartilage (Fig. 1B-E). The Mankin score and osteophyte score for grading OA changes were significantly increased after MIA induction (Fig. 1F and G). After treatment with PDGF-BB for four weeks, the PDGF-BB group showed suppressed cartilage degradation (Fig. 1B-E) and improved Mankin and osteophyte scores compared with those of the OA group (Fig. 1F and G). As shown in Fig. 1A-G, the protective effects of PDGF-BB on cartilage were antagonized by PDGFRi.

PDGF-BB inhibits inflammation in OA chondrocytes induced by MIA in vitro and in vivo.

As an inflammatory disease, the expression of various inflammatory cytokines, such as IL-6, IL-8, TNF- α , iNOS, and COX-2, is increased in OA. Therefore, we evaluated the anti-inflammatory effects of PDGF-BB by ELISA and RT-PCR in vitro and in vivo. The ELISA results showed that PDGF-BB downregulated the concentrations of IL-6, IL-8, TNF- α , iNOS, and COX-2 in both MIA-induced chondrocytes and the OA model (Fig. 2H and Fig. 3A-D). Similarly, the mRNA levels of IL-6, IL-8, TNF- α , iNOS, COX-2, ADAMTS-4, and ADAMTS-5, were upregulated in the OA and OA + PDGF-BB + PDGFRi groups, and these effects were attenuated in the OA + PDGF-BB group (Fig. 2B and Fig. 3E-H). The effects of PDGF-BB were antagonized by PDGFRi. As a result, PDGF-BB treatment inhibited the production of inflammatory factors in both MIA-induced cartilage and cultured chondrocytes at the mRNA and protein levels.

PDGF-BB upregulates the levels of anabolism markers and downregulates the levels of catabolism markers in vitro and in vivo.

During the degradation of cartilage in OA, anabolism markers, such as collagen II, aggrecan, and integrin $\alpha 5\beta 1$, were downregulated, while catabolism markers, such as collagen I, MMPs, and ADATMs, were upregulated. Extracellular matrix anabolism markers, including collagen II and aggrecan, and catabolism markers, including collagen X, MMP-3, MMP-13, and integrin $\alpha 5\beta 1$, were examined by RT-PCR and immunofluorescence staining in vitro and in vivo. The RT-PCR results showed that the mRNA levels of anabolism markers were downregulated and the mRNA levels of catabolism markers were upregulated by MIA in vitro and in vivo (Fig. 2C-D and Fig. 3I-K). The effects of MIA were attenuated by PDGF-BB, and the effects of PDGF-BB were antagonized by PDGFRi (Fig. 2C-D and Fig. 3I-K). Protein expression levels were analyzed by immunofluorescence staining, and the results were similar to the RT-PCR results (Fig. 4, Fig. 5B, C, E, F).

Effects of PDGFR- β regulation on PDGF-BB-mediated chondroprotection

PDGF-BB exerts its effects mainly via PDGFR- β and then initiates signaling. Immunofluorescence analysis was conducted to investigate the activity of PDGFR- β . The results showed low expression of PDGFR- β in cartilage and chondrocytes in the OA and MIA + PDGF-BB + PDGFRi groups compared to the OA + PDGF-BB and control groups (Fig. 5A and D).

Effects of JAK2/STAT, PI3K/AKT, and p38 signaling pathway regulation on PDGF-BB-mediated

chondroprotection in vitro and in vivo

The activation of signaling pathways, such as the JAK/STAT, PI3K/AKT, and MAPK signaling pathways, is involved in the degradation of cartilage through the regulation of targeted proteins and mediates many biological functions in chondrocytes [14–16]. In the current study, we observed MIA-induced upregulation of p-JAK2/p-STAT3, p-PI3K/p-AKT, and p38 in chondrocytes. Therefore, we examined the expression of the phosphorylated proteins mentioned above in PDGF-BB-treated OA by western blotting. In vitro and in vivo, the ratios of p-JAK2/p-STAT3, p-PI3K/p-AKT, and p-ERK1/2 were increased in MIA-induced OA, whereas PDGF-BB treatment downregulated the effects on p-JAK2/p-STAT3, p-PI3K/p-AKT, and p-38 (Fig. 6A-B and 7A-B). The effects of PDGF-BB on the regulation of the JAK2/STAT, PI3K/AKT, and p38 signaling pathways were antagonized by PDGFRi.

PDGF-BB upregulates SOX-9 phosphorylation via PKA activation in vitro and in vivo

The transcription factors SOX-9 and RunX-2 have been proven to significantly influence migration, proliferation, differentiation, and maturation, and RunX-2 in cartilage can also regulate the formation of extracellular matrix[17]. In this study, we investigated the association of PDGF-BB with SOX-9/Runx-2 in SOX-9-shRNA-transfected chondrocytes before PDGF-BB treatment. In vivo, the phosphorylation of SOX-9 was downregulated in the OA group, and the phosphorylation of RunX-2 was upregulated. Upregulated SOX-9 phosphorylation and downregulated RunX-2 phosphorylation were observed after PDGF-BB treatment (Fig. 7C-D). The effects of PDGF-BB on the regulation of SOX-9/RunX-2 were antagonized by PDGFRi and PKAi, respectively (Fig. 7C-D).

Next, we investigated the association of PDGF-BB and SOX-9/RunX-2 in SOX-9-shRNA-transfected chondrocytes before PDGF-BB treatment (Fig. 6C-D, Fig. 7C-D, and Fig. 8). PDGF-BB enhanced the phosphorylation of SOX-9 and PKA and inhibited the phosphorylation of RunX-2 in MIA-induced chondrocytes, and this effect was antagonized by PDGFRi. The effects of PDGF-BB on SOX-9/RunX-2 were attenuated by PKAi and SOX-9-shRNA transfection in MIA-induced chondrocytes, as shown in Fig. 6C-D. Moreover, the inhibition of SOX-9 by SOX-9-shRNA transfection led to increased phosphorylation of RunX-2.

Discussion

The number and activity of chondrocytes are the most important components in cartilage. Various factors can induce the degeneration of chondrocytes and result in damage to cartilage integrity. However, it is difficult for chondrocytes to regenerate themselves and repair the defect site. Many drugs used to treat OA, such as steroids, hyaluronic acid, chondroitin, and nonsteroidal anti-inflammatory drugs (NSAIDs), cannot effectively stop the progression of OA [18]. Accumulating evidence has proven that PDGF-BB has the potential to enhance the proliferation of chondrocytes, which hints at the ability of PDGF-BB to repair damage in OA [8–11]. Therefore, we used PDGF-BB to treat MIA-induced OA, and the

results showed that PDGF-BB attenuated the progression of MIA-induced OA by inhibiting inflammation and enhancing the proliferation of cartilage by regulating the JAK2/STAT3, PI3K/AKT, and p38 signaling pathways, and these effects could be inhibited by a PDGFR inhibitor. Additionally, the upregulation of SOX-9 and the downregulation of RunX-2, which were accompanied by PKA activation, were observed after PDGF-BB treatment in vivo and in vitro. The chondroprotective effects of PDGF-BB on MIA-induced chondrocytes could be partly inhibited by SOX-9-siRNA.

The degeneration of chondrocytes and destructive metabolism of cartilage matrix in articular cartilage are thought to play key roles in the initiation and progression of OA. How to enhance the proliferation of chondrocytes and maintain a stable metabolic environment are research directions in the treatment of OA. A previous study proved that PDGF increased human meniscal cell proliferation and the production of matrix proteins accompanied by increased mRNA expression of collagen II and a three-fold increase in SOX-9 gene transcription [19, 20]. Anita Brandl et al. observed significantly increased proliferation in PDGF-BB-treated human chondrocytes [9]. In IL-1 β -induced human chondrocytes, PDGF-BB inhibited chondrocyte apoptosis and upregulated the levels of collagen type II, cartilage-specific proteoglycans, β 1-integrin and SOX-9 [10]. In the present study, cartilage degeneration was inhibited after MIA induction, as determined by histological changes in cartilage in the PDGF-BB group compared to the NS group. We treated MIA-induced chondrocytes with or without PDGF-BB in vivo and found increased chondrocyte proliferation in these two groups, which was consistent with previous studies [9, 10, 19, 20]. In addition, we also found increases in anabolism markers (aggrecan, type II collagen, and integrin α 5 β 1) and decreases in catabolism markers (MMPS and ADATMS) in PDGF-BB-treated cartilage and chondrocytes. The upregulation of aggrecan, type II collagen, and integrin α 5 β 1 and downregulation of MMPS and ADATMS in our study suggest multiple effects of PDGF-BB on the maintenance of anabolism and catabolism in cartilage in OA.

Osteoarthritis is a chronic degenerative inflammatory process of cartilage in joints, in which cartilage degeneration can stimulate chondrocytes to release inflammatory cytokines, which further damages chondrocytes and accelerates cartilage degeneration [5, 21]. IL-1 is synthesized by abnormal chondrocytes and induces the synthesis of other inflammatory cytokines, such as TNF, IL-6, the chemokine IL-8 and MMPs (MMP 1, MMP 3 and MMP 13), which in turn drive cartilage matrix breakdown and enhance articular chondrocyte destruction [5, 21]. In addition, IL-1 can stimulate the expression of proinflammatory cytokines, such as iNOS and COX-2 [22]. With the abundant accumulation of proinflammatory cytokines, the progression of OA is subsequently exacerbated. Due to its anti-inflammatory potential, PDGF-BB has already exhibited value in the treatment of inflammatory diseases [10, 23]. Therefore, we used PDGF-BB to treat MIA-induced OA and chondrocytes because high expression levels of IL-1, IL-6, IL-8, TNF- α , iNOS, and COX-2 were detected after MIA induction. In the present study, the production of proinflammatory cytokines decreased in the OA + PDGF-BB group and MIA + PDGF-BB group. The results were consistent with studies demonstrating the anti-inflammatory effects of PDGF-BB [10, 23]. Overall, the decreased levels of proinflammatory cytokines proved the anti-inflammatory effect of PDGF-BB on OA- and MIA-induced chondrocytes.

Subsequently, we investigated the potential anti-inflammatory and metabolic mechanisms of PDGF-BB-mediated chondroprotection in OA. PDGF-BB activates PDGFR- β , a receptor tyrosine kinase (RTK), and induces cell proliferation, migration, and angiogenesis by initiating various signaling pathways [24, 25]. PDGFR- β binds to PDGF-BB and remains active following internalization and may thus continue signaling from within endosomes [25]. Subsequent signal transduction and gene regulation were involved in multiple effects. Various studies have proven that the activation of JAK/STAT and PI3K/AKT and the p38 signaling pathway are associated with OA and play important roles in inflammation and the pathogenesis of chondrocyte and extracellular matrix degradation [14, 15]. In view of this important link, we next investigated whether PDGF-BB binding to PDGFR- β participated in the regulation of the JAK2/STAT3, PI3K/AKT, and p38 signaling pathways to prevent OA progression. The activation of PDGFR- β by PDGF-BB was proven in the present study to be accompanied by suppressed activation of JAK2/STAT3, PI3K/AKT, and p38 in MIA-induced OA. Furthermore, chondrocyte proliferation and cartilage anabolism markers, such as aggrecan, collagen II, integrin $\alpha 5\beta 1$, and SOX-9, were significantly increased, while catabolism markers, such as collagen I, MMPs, and ADAMs, were significantly decreased after PDGF-BB treatment. The anti-inflammatory effects of suppressing the activation of JAK2/STAT3, PI3K/AKT, and p38 have already been proven in previous studies [14, 15]. In combination with the results showing that the downregulation of JAK2/STAT3, PI3K/AKT, and p38 was followed by the downregulation of inflammatory cytokines, we concluded that the anti-inflammatory effect of PDGF-BB on OA via the suppression of the JAK2/STAT3, PI3K/AKT, and p38 signaling pathways also played a pivotal role in this process. However, intracellular signal transduction is a complex process and with overlapping interactions. It has been proven that activated JAK2 can regulate PI3K, which indicates that there is crosstalk between JAK2 and the PI3K signaling pathway [26]. In addition, JAK-mediated signaling critically relies on not only STAT transcription factors but also the activation of the MAPK and PI3K/Akt signaling pathways [27]. Furthermore, there is evidence that cAMP-PKA induction blocks inflammatory signaling pathways [28, 29] and that cAMP/PKA activation interacts with JAK2, PI3K and ERK signaling [30–32]. The PKA inhibitor abolished the anti-inflammatory effect of PDGF-BB in the PKAi group compared to the PDGF-BB group, while it did not have a significant influence on the phosphorylation of JAK2/STAT3, PI3K/AKT, or p38. Additionally, a PDGFR- β inhibitor attenuated the activation of PKA in this study. Although we could not identify the order in which JAK, PI3K, p38, and PKA act, PDGF-BB binding to PDGFR- β enhanced chondrocyte proliferation and inhibited inflammation, at least in part due to the downregulation of the JAK2/STAT3, PI3K/AKT, and p38 signaling pathways and the upregulation of PKA. The regulation of JAK, which is an upstream regulatory factor of PI3K/Akt and MAPK, may play a key role in PDGF-BB-mediated chondroprotective effects.

A stable chondrocyte phenotype is essential for maintaining the balance of anabolism and catabolism in cartilage. Hypertrophic chondrocytes are the main cell type in the lesion area in cartilage, in which the SOX-9/RunX-2 balance is a major contributor to the chondrocyte phenotype. RunX-2 is known to be a master transcription factor involved in chondrocyte hypertrophy, is markedly elevated following stimulation and regulates aggrecan loss by upregulating MMP13 and ADAMTS-4/5 expression in OA [33, 34]. Inhibiting RunX-2 leads to decreases in OA severity and the expression of type X collagen and MMP-

13 [35, 36]. Our results were consistent with the previous studies showing that a high increase in RunX-2 in MIA-induced OA and chondrocytes was accompanied by the upregulation of MMP-3, MMP-13, and ADAMTS. In our study, the negative regulation of RunX-2 was reversed by PDGF-BB treatment. It was previously proven that the inhibition of MAPK and the downregulation of RunX-2 to treat OA could be a measurement of degenerative diseases [37]. Combined with the results that PDGF-BB inhibited the activation of the PI3K and p38 signaling pathways in OA, we hypothesized that PDGF-BB induced the downregulation of RunX-2 at least partly via the regulation of the PI3K/AKT and p38 pathways.

SOX-9 acts as a master regulator of aggrecan and collagen and is highly expressed in normal chondrocytes, thus maintaining cartilage and chondrocyte homeostasis [38, 39]. High expression of SOX-9 could inhibit ADAMTS-induced cartilage degeneration, while SOX-9 mRNA expression was reduced in osteoarthritic cartilage [40]. In this study, SOX-9 phosphorylation was suppressed in the OA model, but PDGF-BB induced a positive response by enhancing SOX-9 phosphorylation. Moreover, our results showed that inhibiting PKA activation by a PKA inhibitor and SOX-9 mRNA expression by transfection with siRNA upregulated RunX-2 phosphorylation in chondrocytes. As a result, the downregulation of RunX-2 could be partly mediated by the PKA/SOX-9 signaling pathway.

We next investigated the molecular mechanisms by which PDGF-BB mediates the proliferation of chondrocytes and enhances the phosphorylation of SOX-9. Activated PKA plays an essential role in chondrocyte differentiation, and inhibiting PKA with a PKA antagonist efficiently blocks chondrogenic differentiation [41, 42]. Additionally, PKA-induced SOX9 phosphorylation promoted SOX9 binding to gene enhancer elements and stimulated SOX9 transcriptional activity [43]. With strong evidence of the positive effects of PKA on SOX-9, we sought to explore the underlying mechanism of chondroprotection mediated by PDGF-BB. In this study, we observed increased levels of PKA and SOX-9 in MIA-induced cartilage and chondrocytes in the presence of PDGF-BB. Treatment of MIA-induced chondrocytes with PDGF-BB and a PKA inhibitor suppressed SOX-9 activity, which was also found after treatment with a PDGFR- β antagonist. Consistently, the expression of aggrecan and collagen was upregulated after PDGF-BB treatment. In addition, activation of STAT3 is also sufficient to promote SOX-9 activity for the acquisition of the chondroblast phenotype, whereas inhibiting STAT3 prevents cartilage formation and attenuates SOX9 reporter activity [43]. However, the phosphorylation of STAT3 in this study was significantly increased in MIA-induced OA, which was consistent with previous studies showing that STAT3 activation promoted inflammation and cartilage degradation in OA [16]. A likely explanation is that the main cells in the lesion area are hypertrophic chondrocytes, which tend to undergo apoptosis and lose metabolic capacity compared to normal chondrocytes. Another possibility is that the upstream regulatory pathway may be involved in crosstalk with other proteins induced by inflammation, which in turn suppresses the STAT3-induced activation of SOX-9. Nevertheless, our findings suggested that PDGF-BB enhanced SOX-9 activity, in part, via the PKA signaling pathway and played a critical role in enhancing anabolism in MIA-induced chondrocytes.

Conclusions

As shown in Fig. 9, PDGF-BB exhibited multiple anti-inflammatory and chondroprotective effects in treating OA. The mechanistic analysis provides important insights into our understanding of how PDGF-BB regulates the metabolism of cartilage. Moreover, our findings indicate that PDGF-BB-mediated regulation of SOX-9/RunX-2 serves to promote chondrocyte proliferation and matrix metabolism.

Abbreviations

OA, osteoarthritis; PDGF, platelet-derived growth factor; TNF- α , tumor necrosis factor alpha; IL-1, interleukin 1; MMPs, matrix metalloproteinases; ADAMTS, aggrecanase; COX-2, cyclooxygenase-2; NOSs, nitric oxide synthases; NASIDs, nonsteroidal anti-inflammatory drugs; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; FBS, fetal bovine serum; ELISAs, enzyme-linked immunosorbent assays; RT-PCR, real-time polymerase chain reaction; PDGFR, platelet-derived growth factor receptor; PDGFRi, platelet-derived growth factor receptor receptor inhibitor; RTK, tyrosine kinase.

Declarations

Ethical approval: All experimental animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Ethics Committee for Animal Experimentation of Puai Hospital Affiliated with Huazhong University of Science and Technology.

Consent for publication: Not applicable.

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

Competing interests: The authors declare that they have no competing interests

Funding: This study was funded by the National Natural Science Foundation of Wuhan (Grant NO. WX18Q21 and NO. WX19Q24), the Health Commission of Hubei Province Scientific Research Project (NO. WJ2019H371), the National Natural Science Foundation of Hubei (NO. 2019CFB806)

Author Contributions: YC designed the experiments and directed the study. PZ, ZW, and BL conducted experiments, data analysis and wrote the manuscript. YC and ZS revised the manuscript. All authors read and approved the final manuscript.

Acknowledgment: Not applicable.

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Figures

Figure 1

The in vitro and in vivo effects of PDGF-BB on OA. (A) The viability of chondrocytes was assessed by CCK-8 assays in vitro. PDGF-BB showed cytoprotective effects on OA chondrocytes, and these effects could be antagonized by PDGFRi. (B-G) The effects of MIA, PDGF-BB and PDGFRi on the knees of rats in vivo were assessed by gross observation, X-ray imaging, hematoxylin-eosin staining, and toluidine blue staining. The Mankin score and Osteophyte score were used to grade the changes in cartilage. PDGF-BB exhibited positive effects on OA, and these effects could be attenuated by PDGFRi. *, $p < 0.05$.

Figure 2

The in vitro effects of PDGF-BB on inflammatory cytokines and extracellular matrix. (A) The levels of inflammatory cytokines in chondrocytes were assessed by ELISA. PDGF-BB abrogated the MIA-induced increases in the levels of cytokines. The effects of PDGF-BB were antagonized by PDGFRi. (B) The mRNA levels of inflammatory cytokines in chondrocytes were assessed by RT-PCR. PDGF-BB abrogated the MIA-induced increases in the mRNA levels of cytokines. The effects of PDGF-BB were antagonized by PDGFRi. (C) The mRNA levels of anabolism markers in chondrocytes were assessed by RT-PCR. PDGF-BB abrogated the MIA-induced decreases in the mRNA levels of anabolism markers. The effects of PDGF-BB were antagonized by PDGFRi. (D) The mRNA levels of catabolism markers in chondrocytes were assessed by RT-PCR. PDGF-BB abrogated the MIA-induced increases in the mRNA levels of catabolism markers. The effects of PDGF-BB were antagonized by PDGFRi. *, $p < 0.05$ compared to the control group; **, $p < 0.01$ compared to the control group; ***, $p < 0.001$ compared to the control group.

Figure 3

The in vivo effects of PDGF-BB on inflammatory cytokines, extracellular matrix, and the PDGF receptor. (A-D) The levels of inflammatory cytokines in cartilage were assessed by ELISA. PDGF-BB abrogated the MIA-induced increases in the levels of cytokines. The effects of PDGF-BB were antagonized by PDGFRi. (E-H) The mRNA levels of inflammatory cytokines in cartilage were assessed by RT-PCR. PDGF-BB abrogated the MIA-induced increases in the mRNA levels of cytokines. The effects of PDGF-BB were antagonized by PDGFRi. (I-K) The mRNA levels of anabolism and catabolism markers in cartilage were assessed by RT-PCR. PDGF-BB abrogated the MIA-induced decreases in the mRNA levels of anabolism markers. Moreover, PDGF-BB abrogated the MIA-induced increased in the mRNA levels of catabolism markers. The effects of PDGF-BB were antagonized by PDGFRi. *, $p < 0.05$.

Figure 4

The in vitro effects of PDGF-BB on anabolism and catabolism markers in the extracellular matrix were assessed by immunofluorescence staining. (A-D) PDGF-BB abrogated the MIA-induced decreases in the levels of anabolism markers. The effects of PDGF-BB were antagonized by PDGFRi. (E-L) PDGF-BB abrogated the MIA-induced increases in the levels of catabolism markers. The effects of PDGF-BB were antagonized by PDGFRi. Quantitative data were obtained from fluorescence intensity measurements by

using EN 2009 software (Zeiss). *, $p < 0.05$ compared to the control group; **, $p < 0.01$ compared to the control group; ***, $p < 0.001$ compared to the control group.

Figure 5

The in vivo effects of PDGF-BB on PDGFR-β, collagen II (an anabolism marker), and collagen X (a catabolism marker) in cartilage were assessed by immunofluorescence chemistry. (A and D) PDGF-BB abrogated the MIA-induced decrease in the level of PDGF receptor-β. The effects of PDGF-BB were antagonized by PDGFRi. (B and E) PDGF-BB abrogated the MIA-induced decrease in collagen II. The effects of PDGF-BB were antagonized by PDGFRi. (C and F) PDGF-BB abrogated the MIA-induced increase in the level of collagen X. The effects of PDGF-BB were antagonized by PDGFRi. ***, $p < 0.001$ compared to the control group.

Figure 6

The in vitro effects of PDGF-BB on signaling molecules and transcription factors were assessed by western blotting. (A and B) MIA upregulated the phosphorylation of p38, STAT3, AKT, JAK2, and PI3K in chondrocytes. These upregulated phosphorylated proteins were downregulated by PDGF-BB. The effects of PDGF-BB on the phosphorylation of p38, STAT3, AKT, JAK2, and p38 were antagonized by PDGFRi. (C and D) MIA upregulated the phosphorylation of RunX-2 and downregulated the phosphorylation of PKA and SOX-9 in chondrocytes. Treatment with PDGF-BB downregulated the upregulated p-RunX-2 and upregulated the downregulated p-PKA and p-SOX-9. The effects of PDGF-BB on the phosphorylation of PKA, RunX-2, and SOX-9 were antagonized by PDGFRi and PKAi. PDGF-BB showed no effects on the phosphorylation of RunX-2 and SOX-9 in MIA-treated and SOX-9 siRNA-transfected chondrocytes. Quantitative data were obtained from the results of densitometric methods by using AlphaEaseFC

software (Alpha Innotech). *, $p < 0.05$ compared to the control group; **, $p < 0.01$ compared to the control group; ***, $p < 0.001$ compared to the control group.

Figure 7

The in vivo effects of PDGF-BB on signaling molecules and transcription factors were assessed by western blotting. (A and B) MIA upregulated the phosphorylation of p38, STAT3, AKT, JAK2, and PI3K in chondrocytes. These upregulated phosphorylated proteins were downregulated by PDGF-BB. The effects

of PDGF-BB on the phosphorylation of p38, STAT3, AKT, JAK2, and p38 were antagonized by PDGFRi. (C and D) MIA upregulated the phosphorylation of RunX-2 and downregulated the phosphorylation of PKA and SOX-9 in chondrocytes. Treatment with PDGF-BB downregulated the upregulated p-RunX-2 and upregulated the downregulated p-PKA and p-SOX-9. The effects of PDGF-BB on the phosphorylation of PKA, RunX-2, and SOX-9 were antagonized by PDGFRi and PKAi. Quantitative data were obtained from the results of densitometric methods by using AlphaEaseFC software (Alpha Innotech). *, $p < 0.05$ compared to the control group; **, $p < 0.01$ compared to the control group; ***, $p < 0.001$ compared to the control group.

Figure 8

The in vivo effects of PDGF-BB on RunX-2 and SOX-9 were assessed by immunofluorescence staining. (A-C) The effects of PDGF-BB on the activation of RunX-2. (D-F) The effects of PDGF-BB on the activation of SOX-9. Quantitative data were obtained from the results of densitometric methods by using EN 2009 software (Zeiss). ***, $p < 0.001$ compared to the control group.

Figure 9

Schematic model of the effects of PDGF-BB on OA at the cellular level.