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Glatiramer acetate inhibits degradation of collagen II by suppressing the activity of interferon regulatory factor-1

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ABSTRACT

Pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) is considered to be the major one contributing to the process of development of osteoarthritis (OA).Interferon regulatory factor 1 (IRF-1) is an important transcriptional factor accounting for inflammation response induced by TNF-α. The physiological function of IRF-1 in OA is still unknown. In this study, we reported that the expression levels of IRF-1 in OA chondrocytes were significantly higher compared to those in normal chondrocytes, which was reversed by treatment with Glatiramer acetate (GA), a licensed clinical drug for treating patients suffering from multiple sclerosis (MS). We also found that GA is able to attenuate the upregulation of IRF-1 induced by TNF-α. Matrix metalloproteinase13 (MMP-13) is one of the downstream target genes of IRF-1, which can induce the degradation of collagen II. Importantly, our results indicated that GA suppressed the expression of MMP-13 as well as the degradation of collagen II. In addition, GA also suppressed TNF-α-induced production of NO and expression of iNOS. Finally, we found that the inhibition of STAT1 activation played a critical role in the inhibitory effects of GA on the induction of IRF-1 and MMP-13. These data suggest that GA might have a potential effect in therapeutic OA.

1. Introduction

Osteoarthritis (OA), one of the most common forms of arthritis, is a degenerative disease with low grade inflammation of the joints in aged people [1]. The extracellular matrix components such as collagen and proteoglycan (aggrecan) are important factors for maintaining the integrity of cartilage. 90–95% of the total cartilage collagen is type II collagen, which is the major structural protein in cartilage and is also essentially unique to cartilaginous tissues [2]. In addition, several catabolic factors, including matrix metalloproteinases (MMPs) and nitric oxide (NO) are commonly recognized molecules responsible for joint damage in OA [3]. MMP-13 has been reported to play the greatest part in the pathology of OA degrading the "resident" collagen, especially the type II collagen [4]. Proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) has been considered to play a critical role in the process of development of OA [5]. Interferon response factor-1 (IRF-1) is a transcriptional factor regulating genes induced by interferons, proinflammatory cytokines, and other stimuli and play pivotal roles in immune responses and oncogenesis [6]. IRF-1 has been reported to be an essential regulator of TNF-α stimulus. IRF-1 selectively modulates different sets of genes, including MMP-13 and inducible NO synthase (iNOS) [7]. Increasing evidence has shown that IRF-1 is an essential factor in the pathogenesis of many diseases. However, little information regarding the physiological function of IRF-1 in OA has been reported.

Glatiramer acetate (GA) is a licensed medicine that has been shown to reduce the relapse rate and progression of disability in patients with relapsing remitting MS [8]. Multiple lines of evidence have shown that GA has an anti-inflammation property by increasing the expression of the anti-inflammatory cytokine IL-10 and reducing the expression of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) [9,10]. The findings of anti-inflammatory properties imply that GA might have a potential therapeutic effect in OA. In this study, we reported that GA is able to suppress the transcriptional activity of IRF-1, thereby prevents the induction of MMP-13 as well as the degradation of type II collagen in human chondrocytes.

2. Materials and methods

2.1. Human cartilage samples

All samples were obtained in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical

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Research Involving Human Subjects. The study was approved by the ethics committee of Sun Yat-sen University and Southern Medical University. Written informed consent was obtained from all participants. OA specimens of knee joint cartilage were obtained from the femoral condyle of patients undergoing total knee arthroplasty for primary OA. Normal specimens of knee joint cartilage were obtained from patients with femoral neck fractures who were undergoing femoral head replacement surgeries. The isolated chondrocytes were subsequently cultured in a humid incubator with 5% CO_2 at 37 °C and in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, USA) containing 10% fetal bovine serum (FBS; BioWhittaker, USA) and 100 U/ml of penicillin–streptomycin. Chondrocytes were transfected with non-specific control or STAT1-specific siRNA (100 nM) (Qiagen, Germany) by using Lipofectamine 2000 (Invitrogen, USA) for 24 h. OA chondrocytes were treated with GA (50 μg/ml, TEVA Pharma GmbH, Kirchzarten) for 24 h. Normal human chondrocytes were pretreated with or without GA (50 μg/ml, TEVA Pharma GmbH, Kirchzarten) for 24 h, and were then incubated with TNF-α (10 ng/ml, R&D Systems) for 24 h.

2.2. Real time polymerase chain reaction (PCR)

Total RNA from human chondrocytes was isolated using the Trizol (Invitrogen, USA) according to the manufacturer's protocol. The yield and purity of total RNA were determined by using the absorbance at 260 and 280 nm. 2 μg of the total RNA was reverse transcribed using Superscript First-Strand Synthesis System (Invitrogen, USA). Synthesized CDNA was used for PCR amplification using specific primers, a core kit for SYBR Green (Applied Biosystems, USA) in a Step One Real-Time PCR System (Applied Biosystems, USA). The target mRNA level was normalized to the level of GAPDH and compared with the control. Following primers were used in this study: humanIRF-1: forward, 5′-ctggagagggtctcgct gt-3′; reverse, 5′-ttctggggtcactggtctgt-3′; human MMP-13: forward, 5′-ccagtctccgaggagaaaca-3′; reverse, 5′-aaaaacagctccgcatcaac-3′; human GAPDH: forward, 5′-ccacatcgctcagacaccat-3′; reverse, 5′-cc aggcgcccaatacg-3′.

2.3. Western blot analysis

Human chondrocytes were harvested and resuspended with lysis buffer containing 62.5 mM Tris (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 5% 2-mercaptoethanol, followed by sonication on ice for 30 s. The protein lysates were separated by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PVDF membranes (Bio-Rad, USA). Membranes were blocked with 5% non-fat milk in Tris Buffered Saline with Tween-20 (TBST) for 1 h followed by incubated with primary antibodies at 4 °C overnight. Then membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Dako, USA). Blots were developed using enhanced chemiluminescence according to the manufacturer's protocol (Santa Cruz Biotechnology, USA) [11]. The following antibodies were used in this study: polyclonal antibodies against JAK2, phospho-JAK2, STAT1, p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182) were purchased from Cell Signaling Technology (Beverly, USA). Rabbit polyclonal iNOS, phospho-STAT1 (Tyr701), phospho-Stat1 (Ser727), IRF-1, and β-actin were from Santa Cruz Biotechnology Inc (Santa Cruz, USA). Polyclonal antibodies against collagen II antibodies were purchased from Chemicon International (Temecula, USA).

2.4. Measurement of NO concentrations

The measurement of NO release was performed by the Griess reaction. The concentration of NO was reflected by determination of its stable end product, nitrite, in supernatants [12]. Briefly, a 100 μl aliquot of culture supernatant was incubated with 50 μl of 0.1% sulfanilamide in 5% phosphoric acid and 50 μl of 0.1% N-1-naphthyl-ethylenediamine dihydrochloride. After 10 min of incubation at room temperature, the absorbance was measured at 550 nm with a plate reader measured by using a spectrophotometer.

2.5. Immunofluorescence microscopy

After indicated treatment, cells were fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature (RT). Then cells were permeabilized with 0.4% Triton X-100 followed by blocking with 5% BSA and 2.5% FBS in PBST. Cells were incubated with anti-IRF-1 in PBS with 5% BSA overnight at 4 °C and followed by incubating with TRITC conjugated secondary antibodies for 1 h at RT (Invitrogen, USA). The cells were counterstained with 4′, 6′-diamidino-2-phenylindole dihydrochloride (DAPI) and analyzed on a fluorescence microscope (Olympus DP50).

2.6. Statistical analysis

Experimental values are presented as mean ± S.D. Statistical analysis was evaluated using unpaired Student's *t*-test and one-way analysis of variance (ANOVA) followed by Dunnett's analysis. *P* values less than 0.05 were considered significantly different.

3. Results

We first examined IRF-1 expression in normal and OA chondrocytes by real-time PCR. The results showed that the expression levels of IRF-1 were significantly higher in OA cartilage compared to those in normal cartilage (Fig. 1A). Chondrocytes were stained with IRF-1 antibody. It was shown that IRF-1 was located in the nucleus. Increased IRF-1 level was observed in the nucleus of chondrocytes from OA cartilage (Fig. 1B). Interestingly, treatment with GA inhibited the up-regulation of IRF-1 in chondrocytes from OA (Fig. 1A and B). TNF-α has been reported to play a major role in joint cartilage destruction in OA. Therefore, we checked the effect of GA on the change in IRF-1 expression upon TNF-α treatment. Our results indicated that pretreatment with GA suppressed the increase in IRF-1 induced by TNF-1α in a dose-dependent manner at both mRNA levels (Fig. 1C) and protein levels (Fig. 1D).

MMP-13 is an important target gene of IRF-1. Because MMP-13 is directly responsible for damaging cartilage matrix, we examined the effects of GA on TNF-α-induced increase in MMP-13 activity. The results showed that TNF-α-induced expression of MMP-13 was suppressed by GA at both the mRNA levels (Fig. 2A) and protein levels (Fig. 2B). Since collagen II is preferentially cleaved by MMP-13, we determined whether GA could affect TNF-α-mediated reduction of collagen II. Indeed, GA treatment prevented TNF-α-mediated decrease of collagen II (Fig. 2C). Altogether, the results indicated that GA could provide cartilage protection at least by down-regulating TNF-α-induced MMP-13 expression as well as by preventing TNF-α-mediated decrease of collagen II in chondrocytes.

Previous evidence also implicated IRF-1 as a key regulator of the expression of iNOS and the production of NO. The increased expression of iNOS and production of NO has been reported in OA. When chondrocytes were pretreated with various concentrations of GA, we observed a dose dependent suppression of TNF-α-induced production of NO (Fig. 3A) and expression of iNOS (Fig. 3B).

Regulation of IRF-1 expression is complex. The previous study has demonstrated that signal transducers and activator of transcription 1 (STAT1) plays a critical role in the expression of IRF-1

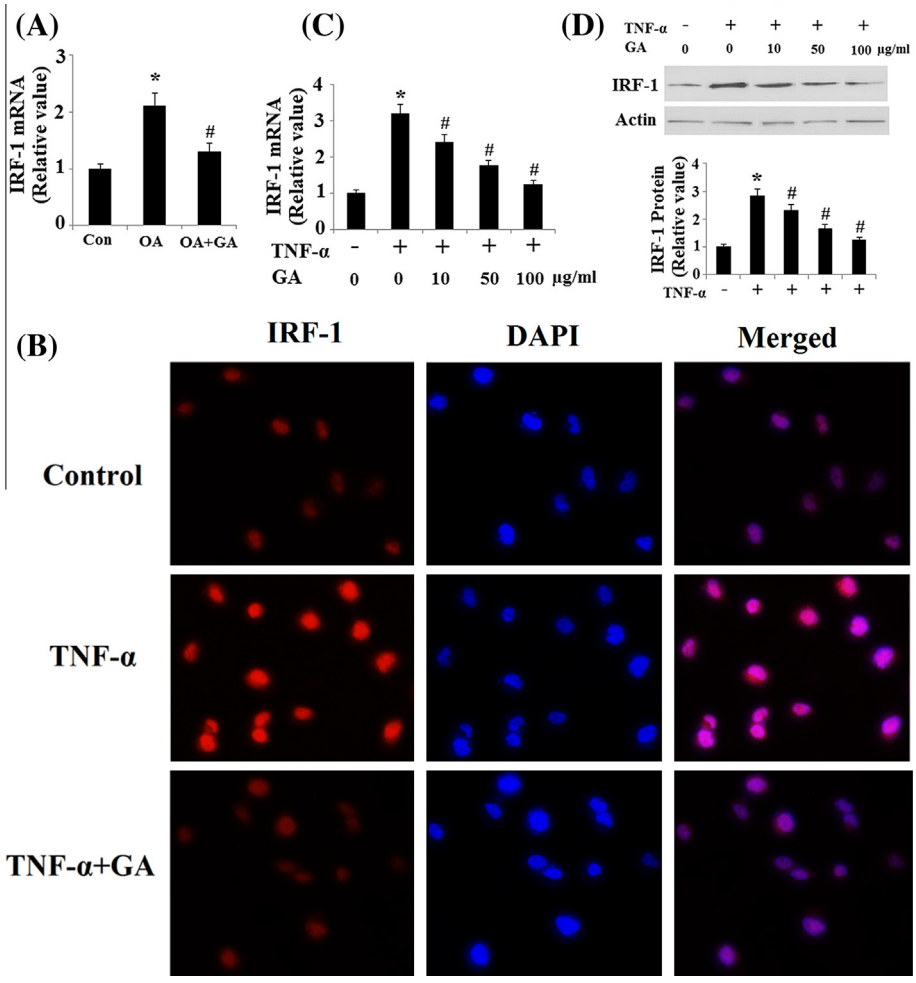

Fig. 1. Glatiramer acetate (GA) attenuates the upregulation of Interferon response factor-1 (IRF-1) in Osteoarthritis (OA) chondrocytes. (A) The expression of IRF-1 at mRNA levels in normal and OA chondrocytes was quantified by real-time PCR. OA chondrocytes was treated with GA ($^{*}P < 0.01$ vs control group; $^{\#}P < 0.01$ vs OA group) for 24 h. (B) The expression of IRF-1 at protein levels in normal and OA chondrocytes was assayed by immunofluorescence. OA chondrocytes was treated with GA for 24 h. (C) Normal human chondrocytes were pretreated with various doses of GA or the solvent, DMSO, for 24 h and then stimulated with TNF-α for 24 h. The expression of IRF-1 was determined at the mRNA level by real time PCR ($^{*}P < 0.01$ vs control group; $^{\#}P < 0.01$ vs TNF treated group). (D) Normal human chondrocytes were pretreated with various doses of GA or the solvent, DMSO, for 24 h and then stimulated with TNF-α for 24 h. The expression of IRF-1 was determined at the protein level by western blot analysis ($^{*}P < 0.01$ vs control group; $^{\#}P < 0.01$ vs TNF treated group).

[13]. Serine phosphorylation of STAT1 at position 727 (S727P) is known to support the interaction of STAT1 with transcriptional coactivators. We investigated the phosphorylated levels of STAT1 at Ser727 in our study. Interestingly, TNF-α treatment increased STAT1 activation, which was attenuated by pretreatment with GA. Tyrosine phosphorylation of STAT1 at amino acid 701 (Tyr701) is an indispensable signature of STAT1 activation. Our results also showed that GA is able to attenuate tyrosine phosphorylation of STAT1 at amino acid 701 (Tyr701) induced by TNF-α. However, the levels of total STAT1 protein did not change. These findings suggest that GA is able to inhibit the activation of STAT1 (Fig. 4A). The STATs transcription factors have been found to be activated by the Janus kinases (JAKs) and to be correlated with p38-MAPK in response of TNF-α. JAK2, is an upstream molecular of STAT1. Therefore, AG490, a JAK2 inhibitor, was used as a negative control for STAT1 activation. Indeed, chondrocytes were treated with TNF-α in the presence or absence of GA and analyzed by western blotting analysis. As shown in Fig. 4B, the TNF-α-induced increase of JAK2 phosphorylation was significantly attenuated by the administration of GA. However, GA treatment didn't change the level of TNF-α-induced increase in phosphorylated p38.We next used siRNA to confirm the role of STAT1 in MMP-13 expression. The successful knockdown of STAT1 was shown in Fig. 4C. Importantly, inhibiting the expression of STAT1 by transfection with STAT1 siRNA reduced the expressions of IRF-1 as well as MMP-13 (Fig. 4D), indicating the principal involvement of STAT1.

4. Discussion

IRF-1 expression is up-regulated in many chronic inflammatory diseases [14] and we firstly demonstrated the elevated expression of IRF-1 in OA chondrocytes. Importantly, we found that treatment with GA mitigated the up-regulation of IRF-1 in OA chondrocytes. In addition to its well-known role in mediating anti-viral responses [14], activation of IRF-1 has been shown to be critical in TNF-α-stimulated NO production [15], as well as the activation of cyclooxygenase-2 (Cox-2) [16]. Only very few reports investigate the role of IRF-1 in chondrocytes. Our observation that GA could suppress TNF-α-induced activation of IRF-1 might suggest its significance in TNF-α-mediated damage of cartilage. Activation of STAT1 by phosphorylation at Ser727 is essential for triggering the expression of IRF-1. Similar with our findings, a recent study has shown that JAK2/STAT1 signaling is involved in MMP-13 induction in IL-1β-treated chondrocytes [17]. Notably, our study indicated that

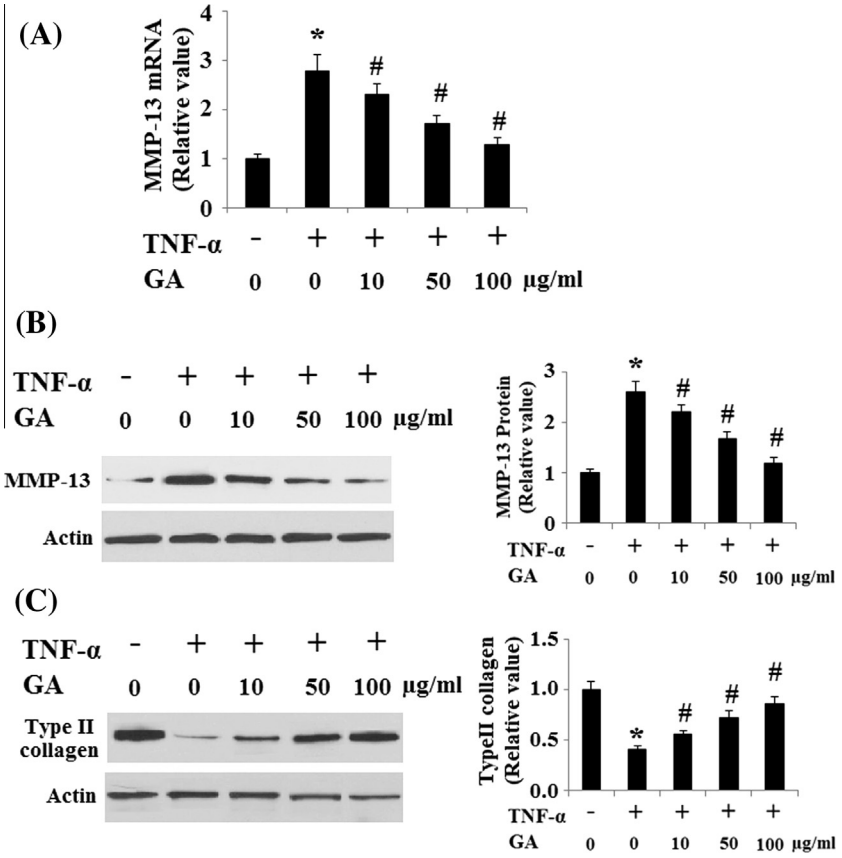

Fig. 2. Glatiramer acetate (GA) attenuates the up-regulation of MMP-13 and the degradation of type II collagen induced by TNF-α in human chondrocytes. Normal human chondrocytes were pretreated with various doses of GA or the solvent, DMSO, for 24 h and then stimulated with TNF-α for 24 h. (A) The expression of MMP-13 was determined at the mRNA level by real time PCR ($^{*}P < 0.01$ vs control group; $^{\#}P < 0.01$ vs TNF treated group). (B) The expression of IRF-1 was determined at the protein level by western blot analysis ($^{*}P < 0.01$ vs control group; $^{\#}P < 0.01$ vs TNF treated group). (C) The levels of collagen II in total cell lysates were determined by Western blot analysis ($^{*}P < 0.01$ vs control group; $^{\#}P < 0.01$ vs TNF treated group).

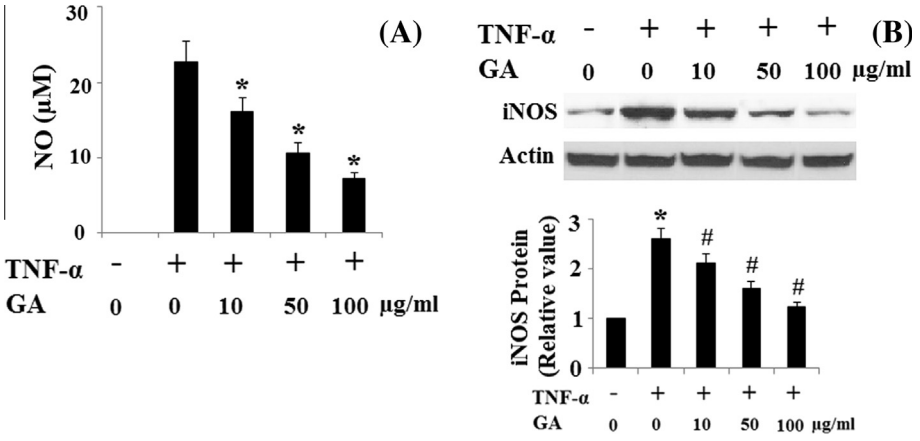

Fig. 3. Glatiramer acetate (GA) attenuates the upregulation of inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO) induced by TNF-α in human chondrocytes. Normal human chondrocytes were pretreated with various doses of GA or the solvent, DMSO, for 24 h and then stimulated with TNF-α for 24 h. (A) Supernatants were collected and the concentrations of NO were determined by Griess reaction ($^{*}P < 0.01$ vs control group; $^{\#}P < 0.01$ vs TNF treated group). (B) The expression of iNOS was determined at the protein level by western blot analysis ($^{*}P < 0.01$ vs control group; $^{\#}P < 0.01$ vs TNF treated group).

pretreatment with GA is able to suppress the induction of IRF-1 by inhibiting the activation of STAT1. This finding was further supported by the observation that knockdown of STAT1 expression by siRNA approach reduced TNF-α- induced IRF-1 and MMP-13 expression.

MMP-13 is an important parameter related to the damage of cartilage in OA, and the induction of which by stimulant TNF-α were chosen as tools to study and to identify potential anti-OA drugs. Both *in vivo* and *in vitro* studies have proved that MMP-13 is critical for OA progression. Pharmacologic inhibition of MMP-13 is an effective strategy to decelerate articular cartilage loss in a murine model of injury-induced knee OA [18]. Different from other MMPs, the expression of MMP-13 is more restricted to connective tissue [19]. Increased activity of MMP-13 led to the

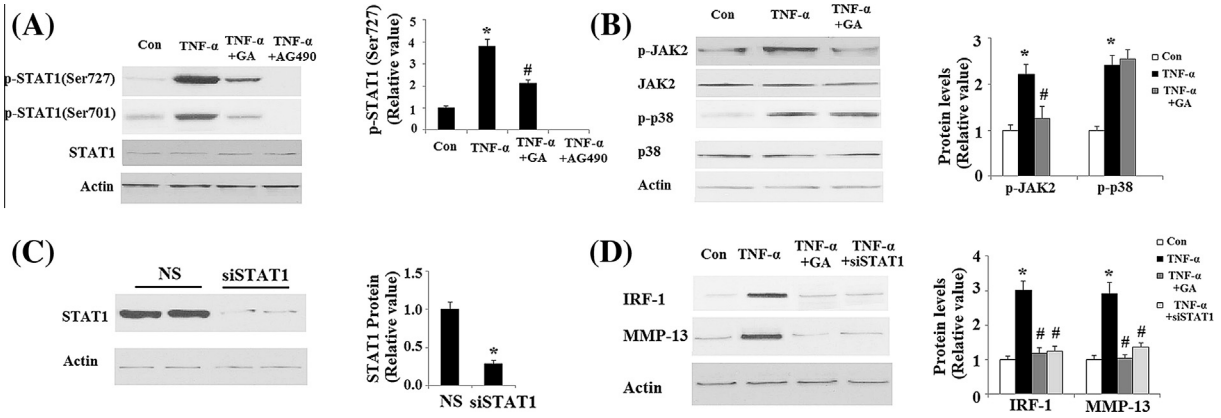

Fig. 4. STAT1 is important for the effect of GA on IRF-1 and MMP-13. (A) Normal human chondrocytes were pretreated with various doses of GA or the solvent, DMSO, for 24 h and then stimulated with TNF-α for 24 h. Immunoblot and quantification analyses revealed that pretreatment with GA mitigated the increased phosphorylation of STAT1 at both Ser727 and Tyr701 (*$P < 0.01$ vs control group; #$P < 0.01$ vs TNF-α treated group). (B) Human chondrocytes were pretreated with or without GA for 24 h, and were then incubated with TNF-α (10 ng/ml) for 24 h. Phospho-JAK2 and phospho-p38 levels were determined by western blot analysis (*$P < 0.01$ vs control group; #$P < 0.01$ vs TNF-α treated group). (C) The expression of STAT1 was knocked down using small RNA interferences, and western blot analysis confirmed the successful knockdown of STAT1 (*$P < 0.01$ vs non-specific (NS) group). (D) Western blot analysis verified that inhibition of STAT1 abolished the inductive effects of TNF-α on IRF-1 and MMP-13 (*$P < 0.01$ vs control group; #$P < 0.01$ vs TNF treated group).

degradation of type-II collagen, which is the basis for articular cartilage. Notably, GA is found to be able to attenuate the effect of TNF-α on the up-regulation of MMP-13 and the degradation of type-II collagen, suggesting a potential role of GA in maintaining the integrity of cartilage. In consistence with our findings, a recent study has shown that GA reverses IL-1β mediated degeneration of articular cartilage via its capacity to induce IL-1Rα expression and inhibit MMP-13 expression and activity, leading to decreased collagen degradation [20]. The production of MMPs is induced by inflammatory cytokines and mediators such as IL-1β, TNF-α and nitric oxide (NO), which are found at elevated levels in OA joint tissues. Both IL-1β [21] and TNF-α [22] could induce the expression of IRF-1. MMP-13 is selectively modulated by IRF-1 [7]. Based on these observations, we speculate that GA can also attenuate the induction of IRF-1 induced by IL-1β. In addition, recent studies also found that GA treatment reduced the production of TNF-α and IL-6 [23]. However, the underlying mechanism is still unknown. Further study will provide us a complete picture.

In addition to degrading type II collagen in cartilage, MMP-13 is also able to degrade proteoglycan, types IV and type IX collagen, osteonectin and perlecan in cartilage. The inhibitory effect of GA on MMP-13 suggests its protective effects on the degradation of these extracellular matrix proteins [24]. Further study will provide a complete understanding on the underlying mechanisms. iNOS–NO is another important parameter related to the damage of cartilage in OA. iNOS is an important target gene of IRF-1 in Chondrocytes [25]. The inhibitory effect of GA on iNOS expression and NO production enhanced our understanding on its pharmacological effects in OA.

In addition to IRF-1, the other two main transcriptional factor pathways regulating TNF-α- induced inflammation are c-Jun and NF-κB [26]. Increased activity of c-Jun [27] and NF-κB [28] has been involved in the pathogenesis of OA. However, little information regarding to the effect of GA on TNF-α inducible genes has been reported before. But it has been reported that GA may inhibit TNF-α-induced RANTES gene expression in human astroglial cells by preventing activation of NF-κB in U-251 MG human astrocytic cells [29]. Therefore, it is suggested that multiple mechanisms might be involved in the inhibitory effect of GA in TNF-α induced inflammation response and degradation of collagen II.

Overall, this study provides evidence of chondroprotective effects and mechanisms of GA, as well as its potential application in TNF-α-induced damage of cartilage in joints. Our approach will therefore be of help in exploring new potential application of licensed clinical drugs in OA. It is anticipated that the results from this report will bring more *in vitro* and *in vivo* studies to confirm the therapeutic benefits of GA in patients with OA and inflammation-mediated joint disorders.

Acknowledgments

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