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Bortezomib prevents the expression of MMP-13 and the degradation of collagen type 2 in human chondrocytes

Weihua Hu, Weikai Zhang, Feng Li, Fengjing Guo, Anmin Chen *

Department of Orthopaedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, PR China

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ABSTRACT

The structural backbone of extracellular matrix in cartilage is the collagen fibril, which is mainly composed of type II collagen. A measurable increase in type II collagen denaturation and degradation has been found in early Osteoarthritis (OA). Pro-inflammatory cytokine such as TNF-α produced in OA cartilage induced the expression of matrix metalloproteinase-13 (MMP-13), which targets and degrades type II collagen. Bortezomib is a proteasome inhibitor approved by the FDA for treatment of multiple myeloma and mantel cell lymphoma. The effects of bortezomib in OA have not been reported before. In this study, we found that bortezomib is able to suppress the degradation of type II collagen induced by TNF-α in human chondrocytes. Mechanistically, bortezomib treatment inhibits the expression of IRF-1 through blunting JAK2/STAT1 pathway, thereby prevents the induction of MMP-13 as well as the degradation of type II collagen. Our findings suggest the therapeutic potentials of bortezomib in patients with OA.

1. Introduction

Osteoarthritis (OA), also known as degenerative arthritis, is a major cause of disability. Gross articular cartilage damage has been considered as the main pathologic feature in OA. Chondrocytes are the only one cell type in articular cartilage, which occupies about 5% of the cartilage volume, and the remainder is occupied by an extensive extracellular matrix [1]. The structural backbone of this matrix is the collagen fibril, which is composed mainly of type II collagen. A measurable increase in type II collagen denaturation with a net loss of this molecule accompanying this damage has been found in early OA [2]. Catabolic activities of OA chondrocytes are related to the elevated release of cartilage degrading enzymes, such as matrix metalloproteinases (MMPs), which mediated the degradation of collagen fibril. Among them, MMP13 is a major enzyme that targets cartilage for degradation. It mainly targets type II collagen in cartilage for degradation [3]. Increasing evidence has demonstrated that high quantities of cytokines such as IL-1β and TNF-α are produced in OA cartilage. TNF-α is a pro-inflammatory cytokine released by synoviocytes, chondrocytes, and invading macrophages in inflamed joints. JAK/STAT pathway, specifically JAK2/STAT1/2, is revealed to be involved in MMP-13 [4]. Transcriptional factor interferon response factor-1 (IRF-1), the downstream molecular of JAK2/STAT1/2, has been considered a critical factor mediating the expression of MMP-13 induced by interferons, proinflammatory cytokines, and other stimuli [5]. Inhibition of type II collagen breakdown though preventing its upstream signals has been considered as a potential pharmacological strategy for OA treatment.

Bortezomib, a proteasome inhibitor approved by the FDA for treatment of multiple myeloma and mantel cell lymphoma, acts by targeting the catalytic 20S core of the proteasome and induces apoptosis in cancer cells [6,7]. The efficacy of bortezomib for the treatment of solid tumors has been investigated by a number of clinical trials [8]. Interestingly, bortezomib was found to inhibit the activation of NF-κB, which in turn, down-regulates the expression of the associated inflammatory genes, including interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, interferon (IFN)-c, monocyte chemoattractant protein (MCP)-1, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, inducible nitric oxide synthase (iNOS), and cyclo-oxygenase (COX)-2 [9]. The findings of anti-inflammatory properties imply that bortezomib might have a potential therapeutic effect in OA. In this study, we reported that bortezomib is able to suppress the degradation of type II collagen in human chondrocytes. Mechanistically, bortezomib treatment inhibits the expression of IRF-1, thereby prevents the induction of MMP-13 as well as the degradation of type II collagen.

* Corresponding author. Address: Department of Orthopaedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan 430030, PR China. Tel.: +86 27 83665318.
E-mail address: anchen066@yeah.net (A. Chen).

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2. Materials and methods

2.1. Human cartilage samples

Human subject studies were according to the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. In addition, experimentation protocols were submitted to and approved by the ethics committee of Huazhong University of Science and Technology. Written informed consent was obtained from all research participants. Samples from these femoral heads, which were obtained from patients undergoing prosthetic replacement due to fracture of the femoral neck distal femoral tumor were used to isolate chondrocytes. Isolated chondrocytes were maintained in Dulbecco's modified Eagle's (DMEM) medium (GIBCO-BRL, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humid incubator with 5% CO_2 at 37 °C. Normal human chondrocytes were pretreated with or without bortezomib at the concentrations of 2.5, 5, 10 μM for 24 h, followed by incubated with TNF-α (5 ng/ml, R&D Systems) for another 24 h.

2.2. Real-time polymerase chain reaction (PCR)

Total RNA from cells was isolated by using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA samples were treated with DNase I (Roche, Indianapolis, IN, USA) for 15 min at RT before reverse transcription. 2 mg of total RNA was used for reverse transcription based on the description of applying Superscript First-Strand Synthesis System (Invitrogen). The obtained template cDNA samples were subjected to real time PCR reactions by using power SYBR Green PCR Master Mix (Applied BioSystems, Foster City, CA, USA) according to the manufacturer's instructions.

2.3. Fluorescence staining of IRF-1

After indicated treatment, cells grown on plain coverslips were fixed in 4% paraformamide at RT for 15 min and permeabilized with 0.4% Triton X-100 on ice for 10 min. Then cells were blocked with 5% BSA and 2.5% FBS in PBST, followed by incubated with rabbit anti-human p65 antibody. The coverslips were then washed and the cells were incubated with Alexa-594 conjugated secondary antibodies for 1 h at RT. The coverslips were mounted and observed under a fluorescence microscope.

2.4. Immunoblotting analysis

Cells were lysed by cell lysis buffer (cell signaling, USA). Protein concentration was determined by the bicinchoninic acid assay (BCA assay). 20 μg total proteins were resolved in 4–15% gradient TRIS–HCl gels (BIORAD, USA). After gel electrophoresis, the proteins were transferred to the polyvinylidene difluoride (PVDF) membrane [10]. The membranes were blocked with 5% fat-free dry milk in PBST (1× PBS with 0.1% Tween-20) for 1 h at room temperature (RT), followed by an overnight incubation at 4 °C with primary antibodies in PBST with 2% fat-free milk. The membranes were then incubated with horseradish peroxidase labeled anti-mouse or anti-rabbit secondary antibodies for 1 h at RT. Immunoreactivity was detected using Beyo ECL Plus reagent (enhanced chemiluminescence).

2.5. Statistical analysis

Statistical analysis was performed using the one-way analysis of variance (ANOVA). Experimental data are presented as mean ± S.D. values of $P < 0.05$ were considered to indicate statistically significant differences.

3. Results

Bortezomib is the first therapeutic proteasome inhibitor to be tested in humans, the molecular structure of which is shown in Fig. 1A. Cells were stimulated with TNF-α in the presence or absence of several different doses of bortezomib. By Western blotting, the results showed that TNF-α decreased collagen II levels, which can be prevented by bortezomib in a dose dependent manner (Fig. 1B). MMPs, especially MMP-13 has been reported to play the greatest part in the pathology of OA degrading the "resident" type II collagen. By RT-PCR, the results showed that TNFα-induced expression of MMP-13 (Fig. 2A) at mRNA levels, which was inhibited by treatment with bortezomib. Consistently, Western blot analysis revealed that bortezomib inhibits the expression of MMP-13 (Fig. 2B) at protein levels.

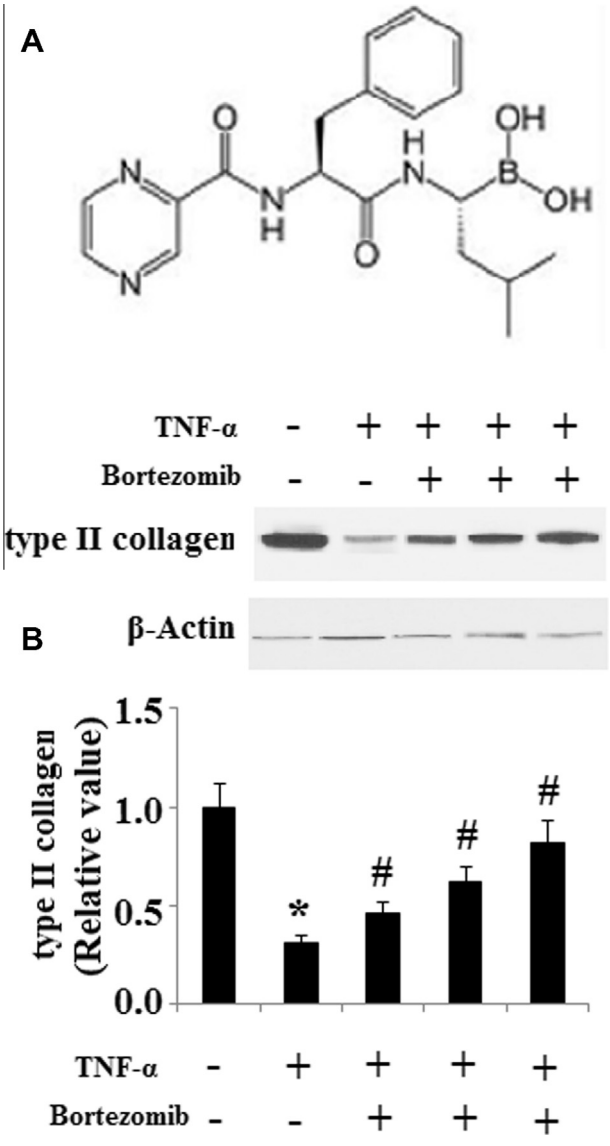

Fig. 1. Impact of bortezomib on degradation of collagen type II induced by TNF-α in human chondrocytes. (A) Molecular structure of bortezomib; (B) human chondrocytes were pretreated with bortezomib at a variety of concentrations (2.5, 5, 10 μM) for 24 h followed by treated with TNF-α (5 ng/ml) for another 24 h. The levels of collagen type II were determined by Western blot analysis ($^{*}P < 0.01$ vs control group; $^{\#}P < 0.01$ vs TNF-α treated group).

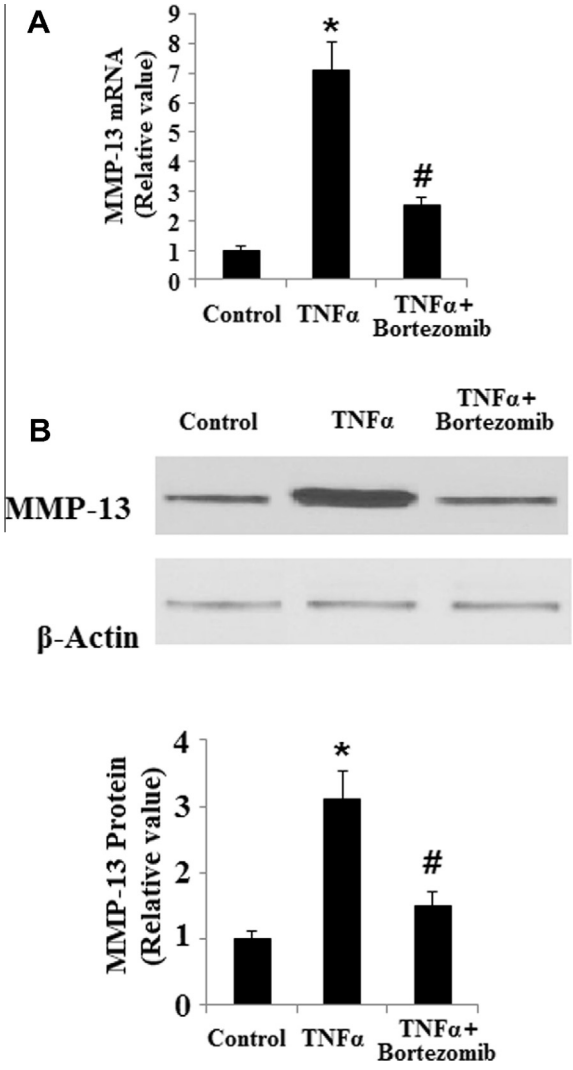

Fig. 2. Bortezomib mitigates the expression of MMP-13 induced TNF-α. Normal human chondrocytes were pretreated with bortezomib (5 μM) or the solvent, DMSO, for 24 h and then stimulated with TNF-α (5 ng/ml) for another 24 h and (A) the expression of MMP-13 at the mRNA level was determined by real time PCR (*$P < 0.01$ vs control group; #$P < 0.01$ vs TNF-α treated group). (B) The expression of MMP-13 at the protein level was determined by Western blot analysis (*$P < 0.01$ vs control group; #$P < 0.01$ vs TNF-α treated group).

STATs have been reported to play an essential role in MMP-13 expression. And TNF-α is reported to increase JAK2 as well as down-stream STAT1 activation. We then examined the effects of bortezomib on the activity of STAT1. Our results indicate that bortezomib inhibits the activation of STAT1 induced by TNF-α treatment. AG490, a JAK2 inhibitor, was used as a positive control (Fig. 3A). Importantly, AG490 also inhibits the expression of MMP-13 induced by TNF-α treatment (Fig. 3B).

Transcriptional factor IRF-1 mediates gene induction down-stream of STAT1. Notably, IRF-1 is involved in the expression of MMP-13. Thus, we examined the effects of bortezomib on the expression of IRF-1. As shown in Fig. 4A, real time PCR results indicate that TNF-α treatment increases the expression of IRF-1, which is inhibited by bortezomib at mRNA levels. AG490 was used as a positive control. Immunocytochemistry analysis reveals that bortezomib attenuates the expression of IRF-1 induced by TNF-α treatment (Fig. 4B).

4. Discussion

The extracellular matrix components such as collagen and proteoglycan (aggrecan) are essential for maintaining the integrity of cartilage [11]. As a major articular cartilage constituent, Type II collagen represents 90–95% of its total collagen content and forming the fibrils that give cartilage its tensile strength. A measurable degradation and loss of this molecule has been found in early OA [12]. Several chemical signals have been revealed to drive this process. A wide variety of molecular fragments are produced as a result of the extensive proteolysis during the process of matrix degradation. These signals induce the expression and secretion of MMPs and prodegradative cytokines such as IL-1 and TNF-α, which appear to have a key role in cartilage degeneration in OA. Both these cytokines and their receptors are upregulated in chondrocytes [13]. TNF-α has been reported to induce the expression of MMP-13 through increasing the expression and transcriptional capacity of IRF-1 mediated by the JAK1/STAT1/2 pathway [14]. Our observation that bortezomib could suppress TNF-α-induced activation of IRF-1 might suggest its significance in TNF-α-mediated damage of cartilage. Phosphorylation at Ser727 plays an essential role for triggering the expression of IRF-1. Similar with our findings, Lim and colleagues found that JAK2/STAT1 signaling is involved in MMP-13 induction in IL-1β treated chondrocytes [15]. Importantly, our findings indicate that bortezomib ameliorates the up-regulation of MMP-13 and the degradation of type-II collagen induced by TNF-α, suggesting a potential role of bortezomib in maintaining the integrity of cartilage.

Bortezomib is the first proteasome inhibitor to display anticancer activity in both solid and hematological malignancies [16]. The nuclear factor NF-κB pathway has long been considered a prototypical proinflammatory signaling pathway. Notably, administration of bortezomib increases the accumulation of phosphorylated IκBα, thereby decreases nuclear translocation of NF-κB. Thus, NF-κB signaling and activation of its downstream targets such as

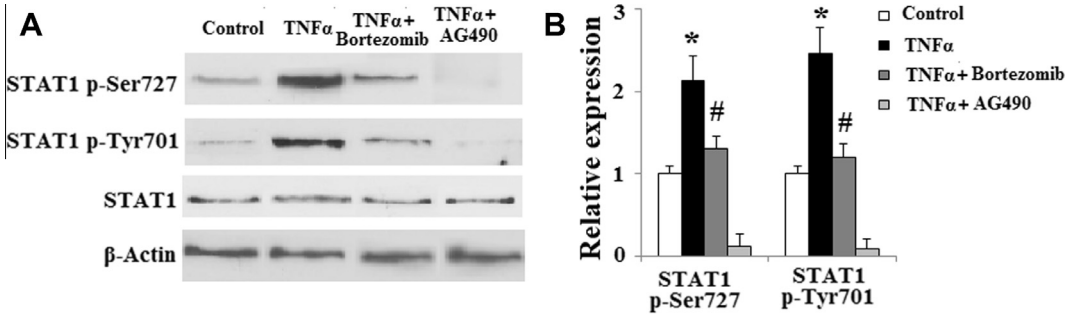

Fig. 3. Bortezomib prevents the activation of STAT1. Normal human chondrocytes were pretreated with bortezomib (5 ng/ml) or the solvent, DMSO, for 24 h and then stimulated with TNF-α (5 ng/ml) for another 24 h. Immunoblot and quantification analyses revealed that pretreatment with bortezomib mitigated the increased phosphorylation of STAT1 at Ser727 and Tyr701; (A) representative immunoblot bands; (B) quantification analyses (*$P < 0.01$ vs control group; #$P < 0.01$ vs bortezomib treated group).

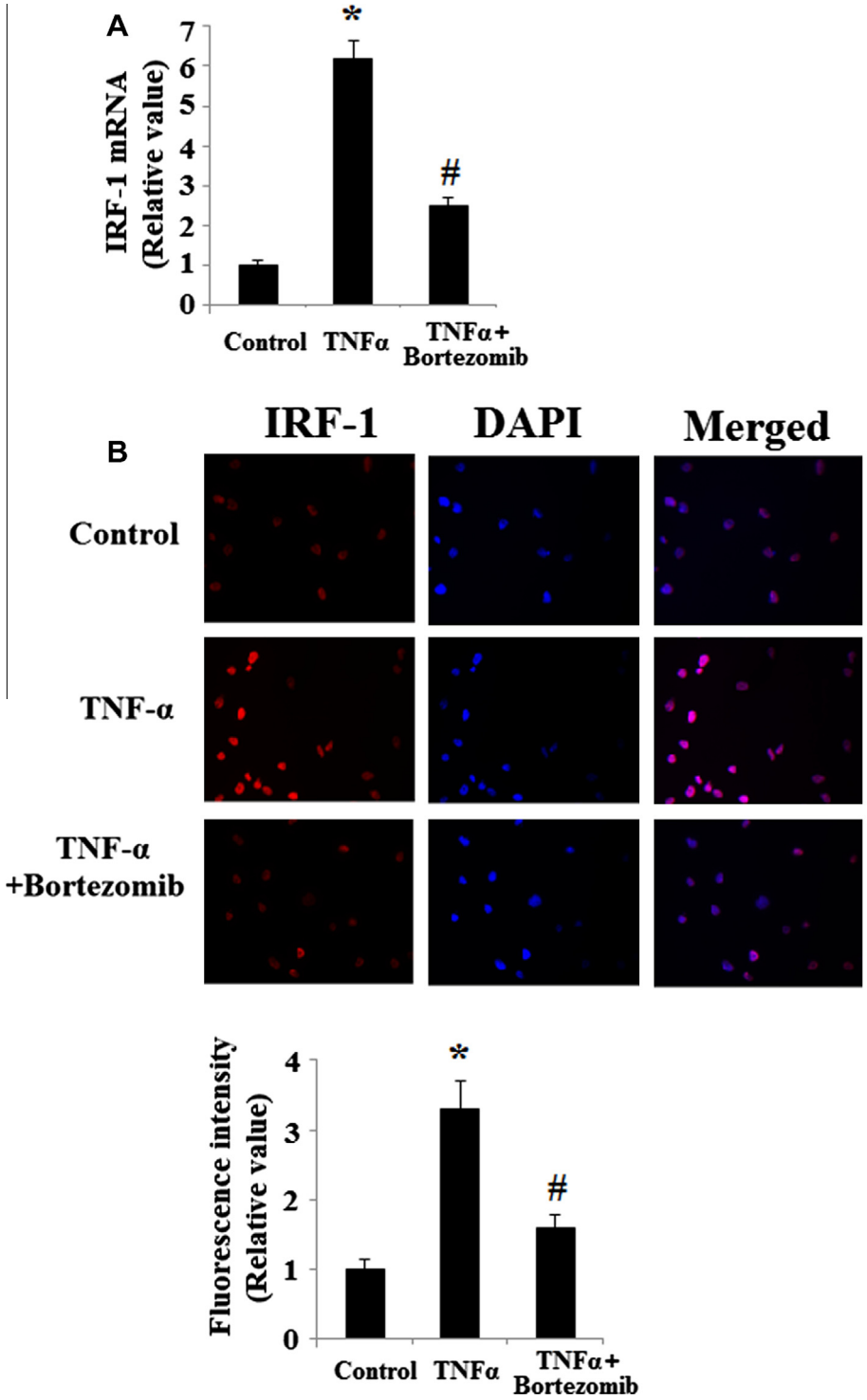

Fig. 4. Bortezomib ameliorates the upregulation of interferon response factor-1 (IRF-1) induced by TNF-α. Normal human chondrocytes were pretreated with bortezomib (5 μM) or the solvent, DMSO, for another 24 h and then stimulated with TNF-α (5 ng/ml) for 24 h and (A) the expression of IRF-1 at the mRNA level was determined by real time PCR ($^{*}P < 0.01$ vs control group; $^{\#}P < 0.01$ vs TNF-α treated group). (B) The expression of IRF-1 at the protein level was determined by immunofluorescence.

IL-1, TNF-α are suppressed. These results suggest that bortezomib exhibits anti-inflammatory activity [17]. Consistently, another study displayed that treatment with bortezomib inhibits the expression of NF-κB downstream signaling molecules, such as IL-6, VEGF, IL-8, cIAP1, cIAP2, XIAP and FLIP [18]. In this study, we found that bortezomib inhibits the induction of MMP-13 caused by TNF-α. MMP-13 is selectively modulated by IRF-1. Upregulation of IRF-1 expression has been found in many chronic inflammatory diseases [19]. Notably, the elevated expression of IRF-1 was found in OA chondrocytes [20]. JAK/STAT signaling pathways are involved in collagen II degradation as well as the activation of MMPs [21]. Our results indicate that the inhibitory effects of bortezomib on MMP-13 expression and degradation of collagen II is mediated by the JAK/STAT/IRF-1 pathway. These findings suggest multiple

mechanisms might be involved in the inhibitory effect of bortezomib in TNF-α induced inflammation response and degradation of collagen II.

Acknowledgments

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