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The protective effects of Donepezil (DP) against cartilage matrix destruction induced by TNF-α

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ABSTRACT

The extracellular matrix apparatuses containing collagen and proteoglycan (aggrecan) are important factors for maintaining the integrity of cartilage. Collagen type II, the main component of total cartilage, is mainly degraded by matrix metalloproteinase13 (MMP-13), which is an important molecule responsible for joint damage in Osteoarthritis (OA). Donepezil (DP), a potent and selective acetylcholinesterase inhibitor, is a medication approved by the US Food and Drug Administration and used in the alleviation of dementia in Alzheimer's disease (AD). In this study, we found that DP treatment prevented the degradation of collagen type II induced by TNF-α. Mechanistically, DP treatment leads to the inhibition of the transcriptional activity of interferon response factor-1 (IRF-1), thereby prevents the induction of MMP-13. These findings suggest the potential therapeutic effects of DP in OA.

1. Introduction

Osteoarthritis (OA) is one of the most common forms of arthritis. Studies have reported that inflammation plays a pivotal role in the development and progression of OA. The extracellular matrix apparatuses containing collagen and proteoglycan (aggrecan) are important factors for maintaining the integrity of cartilage. Among them, collagen type II is the main component of total cartilage, which is essentially unique to cartilaginous tissues [1]. Collagen type II is mainly degraded by matrix metalloproteinase13 (MMP13), which is an important molecule responsible for joint damage in OA [2]. Secretion of pro-inflammatory cytokines such as TNF-α and IL-1β in OA stimulates the expression of MMPs. Notably, interferon response factor-1 (IRF-1), a transcriptional factor induced by interferons, proinflammatory cytokines, and other stimuli has been shown to mediate the expression of MMPs [3]. Exploring potential pharmacological targets for inhibition of type II collagen breakdown and its upstream molecules has been considered as important strategy for OA treatment.

Donepezil (DP), a potent and selective acetylcholinesterase inhibitor new class of acetylcholinesterase inhibitor, is the second drug approved by FDA for treatment of mild to moderate Alzheimer's diseases (AD) [4,5]. DP has been shown to provide neuroprotection by exerting its anti-inflammatory effects through inhibiting the production of interleukin-1β (IL-1β), IL-6, IL-18 and monocyte chemoattractant protein 1 (MCP-1), and suppressing microglial activation which was independent of acetylcholine and its receptor [6,7]. In addition, DP also could increase the IL-4 level and expression and reverse the AD-related down-regulation of the IL-4/MCP-1 axis [8]. The findings of anti-inflammatory properties imply that DP might have a potential therapeutic effect in OA. In this study, we reported that DP is able to suppress the degradation of type II collagen in human chondrocytes. Mechanistically, DP treatment leads to inhibition of the transcriptional activity of IRF-1, thereby prevents the induction of MMP-13.

2. Materials and methods

2.1. Human cartilage samples

Human subject researches were approved by the ethics committee of Third Military Medical University. All of the participants have signed the written informed consent. Studies were guided by the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. Briefly, 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 cultured in an incubator with 5% CO_2 at 37 °C and in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (P/S). Normal human chondrocytes were pretreated with or without DP at the concentrations

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of 10 or 20 μM for 24 h, followed by incubated with TNF-α (10 ng/ml) for another 24 h.

2.2. Western blot analysis

Western blot analysis was performed to determine the expression of proteins in this study. Briefly, proteins were extracted from cultured cells by using cell lysis buffer (Cell signaling, USA). Then samples were loaded on 10% gradient sodium dodecyl sulfatepolyacrylamide gels (SDS–PAGE). Separated proteins were transferred onto a PVDF membrane (Bio-Rad, USA). The membranes were blocked by 5% non-fat milk. Afterward, the membranes were incubated with primary antibodies overnight at 4 °C. After 3 times washes with TBST buffer, the membranes were incubated with anti-mouse-HRP and goat anti-rabbit-HRP for 30 min respectively at room temperature (RT) [9]. β-actin was used as internal control. The bands were quantified with the image J software.

2.3. RNA isolation and real-time PCR

Total RNA from cultured cells was isolated using Trizol (Invitrogen, USA) following the manufacturer's instructions. 2 μg of the total RNA was used for first-strand cDNA by using SuperScript III reverse transcriptase, oligo(dT), and random primers (Invitrogen). Real-time PCR was carried out using TaqMan gene expression assays (Applied Biosystems, USA) in a StepOne Plus Real time PCR System. Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $_{\Delta\Delta}$Ct method.

2.4. Immunocytochemistry

After indicated treatments, human chondrocytes were fixed with 4% paraformaldehyde at room temperature (RT) for 15 min. After washed for 3 times, cells were permeabilized in 0.1% triton-100 solution for 10 min on ice, followed by blocked with 10% goat serum at RT for 30 min. Then cells were sequentially incubated for 2 h with primary antibodies at RT and Alexa 488-conjugated anti-mouse secondary antibody (1:200 dilution) for 1 h at RT. After mounted in Fluoromount medium, immunofluorescent signals in slides were captured on a fluorescence microscope.

2.5. Statistical analysis

All data are presented as means ± S.E.M resulting from at least three independent experiments. Statistical analysis was performed using one-way variance of analysis (ANOVA). A significant difference was considered at $P < 0.05$.

3. Results

Donepezil (DP) is a potent and selective acetylcholinesterase inhibitor, the molecular structure of which is shown in Fig. 1A. Our results indicated that TNF-α treatment decreased the level of collagen type II. Interestingly, DP treatment prevented the degradation of collagen type II induced by TNF-α in a dose dependent manner (Fig. 1B).

Since collagen II is preferentially cleaved by MMP-13, we then examined the effects of DP on the expression of MMP-13. Real time PCR result showed that TNF-α-induced expression of MMP-13 was suppressed by DP at both the mRNA levels (Fig. 2A) and the protein levels in a dose dependent manner (Fig. 2B). The transcriptional factor IRF-1 is a main regulator of MMP-13 transcription. Therefore, we examined the effect of DP on the change in IRF-1 expression upon TNF-α treatment. Real time PCR results demonstrated that pretreatment with DP suppressed the increase in IRF-1 induced by TNF-α in a dose-dependent manner at mRNA levels (Fig. 3A). The results were confirmed at the protein levels by the immunofluorescence study (Fig. 3B). IRF-1 expression is controlled by the signal transducers and activator of transcription 1 (STAT1). And serine phosphorylation of STAT1 at position 727 (S727) and tyrosine phosphorylation of STAT1 at position 701 (Tyr701) are two important phosphorylation sites regulating the activation of STAT1. Our results indicated that TNF-α treatment increased the phosphorylated levels of both S727 and Tyr701, which was ameliorated by pretreatment with DP (Fig. 4A–C). These findings suggest that DP might prevent the expression of IRF-1 and MMP-13 through inhibiting the activation of STAT1.

4. Discussion

DP is a centrally acting reversible acetylcholinesterase inhibitor, the main therapeutic use of which is in the palliative treatment of Alzheimer's disease (AD). However, the precise mechanism of

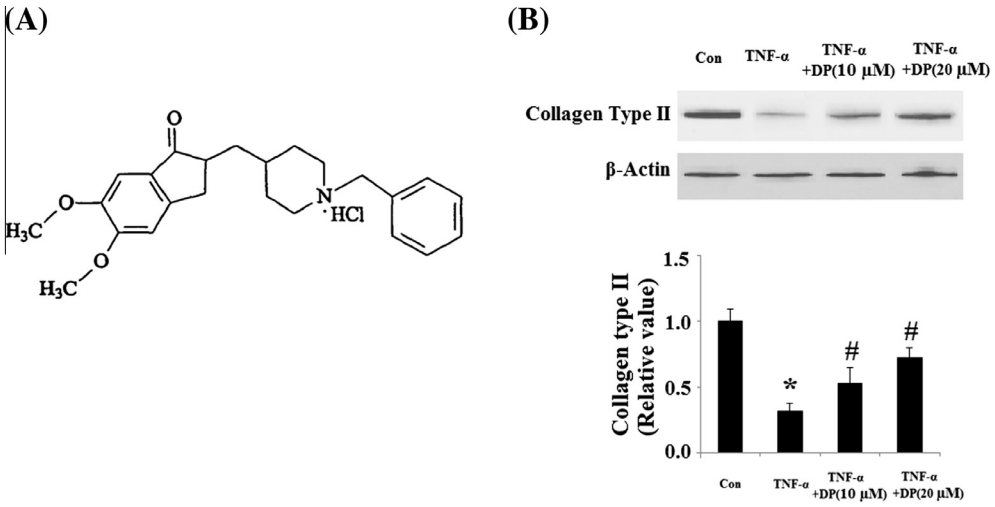

Fig. 1. Donepezil (DP) ameliorates the degradation of collagen type II induced by TNF-α in human chondrocytes. (A) Molecular structure of Donepezil (DP); (B) the levels of collagen type II in total cell lysates were determined by Western blot analysis (*$P < 0.01$ vs control group; #$P < 0.01$ vs TNF-α treated group).

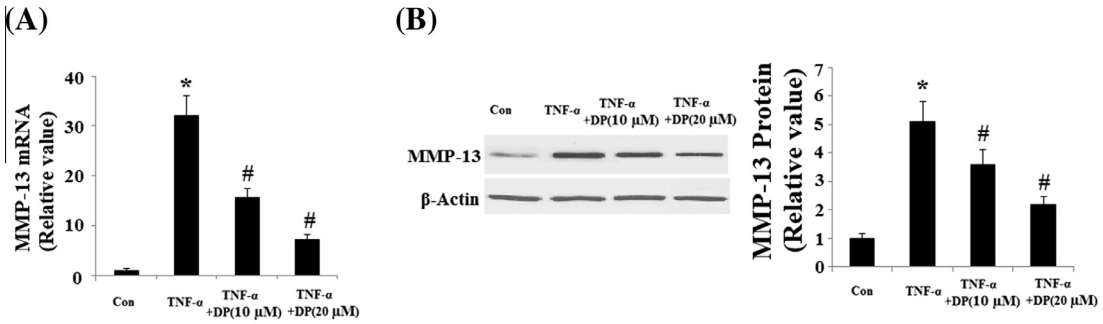

Fig. 2. Donepezil (DP) mitigates the expression of MMP-13 induced by TNF-α in human chondrocytes. Normal human chondrocytes were pretreated with DP (10 μM) or the solvent, DMSO, for 24 h and then stimulated with TNF-α (10 ng/ml) for another 24 h and (A) the expression of MMP-13 at the mRNA level was 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).

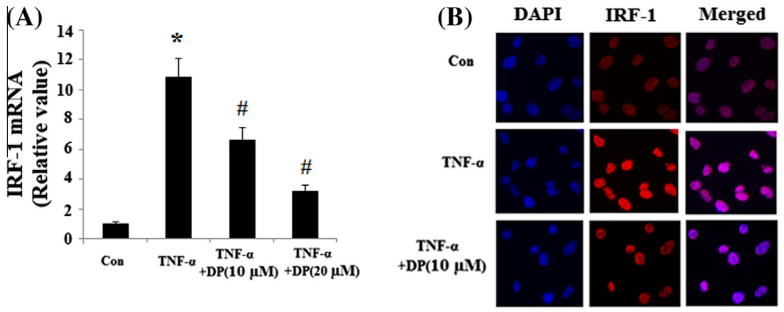

Fig. 3. Donepezil (DP) attenuates the upregulation of interferon response factor-1 (IRF-1) induced by TNF-α. Normal human chondrocytes were pretreated with Donepezil (DP) or the solvent, DMSO, for 24 h and then stimulated with TNF-α (10 ng/ml) for another 24 h and (A) the expression of IRF-1 at the mRNA 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.

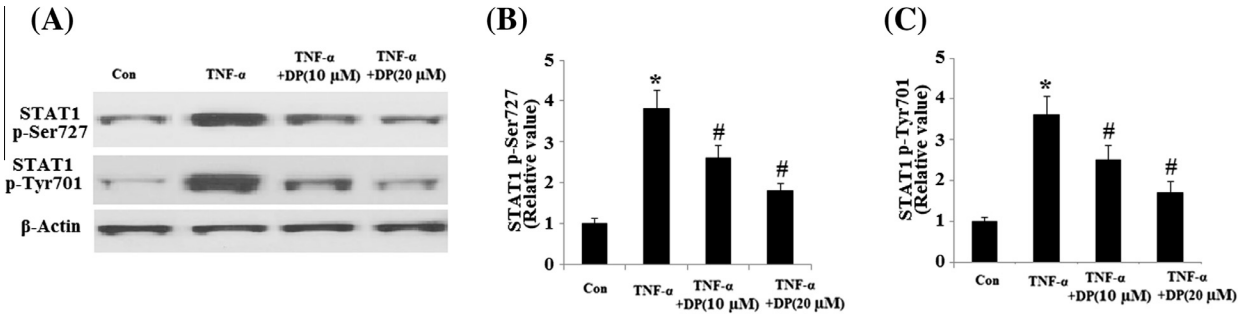

Fig. 4. Donepezil (DP) prevents the activation of STAT1. Normal human chondrocytes were pretreated with Donepezil (DP) (10 or 20 ng/ml) or the solvent, DMSO, for 24 h and then stimulated with TNF-α (10 ng/ml) for another 24 h. Immunoblot and quantification analyses revealed that pretreatment with Donepezil (DP) mitigated the increased phosphorylation of STAT1 at Ser727 and Tyr701; (A) representative immunoblot bands; (B), (C) quantification analyses (*$P < 0.01$ vs control group; #$P < 0.01$ vs TNF-α treated group).

activity of DP is still unclear. Our results of examination in human chondrocytes *in vitro* indicated that the DP suppresses TNF-α-mediated the induction of MMP-13 occurred through inactivating STAT1/IRF-1, thereby preventing collagen type II degradation, suggesting a potential use of DP in OA.

90–95% of the total cartilage collagen is collagen type II, which is the major structural protein in cartilage and is also essentially unique to cartilaginous tissues [10]. The degradation and loss of collagen type II is an important parameter related to the damage of cartilage in OA, mainly caused by MMP-13. MMP-13 is a major enzyme that targets cartilage for degradation. Compared to other MMPs, the expression of MMP-13 is more restricted to connective tissue [11]. The induction of MMP-13 by stimulant TNF-α has been considered as an important tool to study and to identify potential anti-OA drugs. Notably, inhibiting the expression and activity of MMP-13 is an effective strategy to decelerate articular cartilage loss. In addition to degrading collagen type II in cartilage, MMP-13 is also able to degrade proteoglycan, types IV and type IX collagen, osteonectin and perlecan in cartilage [12]. The inhibitory effect of DP on MMP-13 suggests its protective effects on the degradation of these extracellular matrix proteins. MMP-13 is regulated by IRF-1.

IRF-1 expression is up-regulated in many chronic inflammatory diseases. Importantly, it has been demonstrated to be elevated in OA chondrocytes [13]. Activation of STAT1 is essential for triggering the expression of IRF-1. Consistent with our findings, JAK2/

STAT1 signaling has been reported to be involved in MMP-13 induction in IL-1β treated chondrocytes [14]. Our result indicated that pretreatment with DP is able to suppress the induction of IRF-1 by inhibiting the activation of STAT1. As a matter of fact, the underlying mechanisms by which DP exerts its beneficial effects may be multifactorial. It has been reported that DP could exert its anti-inflammatory effects through inhibiting the production of interleukin-1β (IL-1β), IL-6, IL-18, which could induce the degradation of collagen type II [15].

In addition, DP particularly dampens the activation of macrophages [16]. With regarding to the effects of DP on MMPs, DP has been reported to reduce the permeability of blood brain barrier (BBB) by inhibiting the expression of MMP-2 and MMP-9 [17]. MMPs are also transcriptionally regulated by NF-κB, another important inflammation regulator. Notably, DP has been reported to inhibit NF-κB activation [18].

Taken together, our findings provide evidence of chondroprotective effects and mechanisms of DP, as well as its potential application in TNF-α-induced damage of cartilage in joints. However, the influence of DP on preventing collagen type II degradation mechanisms is not fully understood. Further research will provide us with a more complete picture of the underlying mechanisms of chondroprotective effects of DP in OA.

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