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Original Article

Effects of $1,25(OH)_2D_3$ on cell proliferation, apoptosis, and expression of SOST, β-catenin, Collagen II, and MMP13 in rat articular chondrocytes induced by TNF-α *in vitro*

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Abstract: The aim of the current study was to observe the effects of TNF-α on proliferation and apoptosis of rat articular chondrocytes, as well as the protective effects of $1,25(OH)_2D_3$ on chondrocytes induced by TNF-α. TNF-α levels in patients with osteoarthritis (OA) were measured by ELISA. Articular chondrocytes, isolated from rat knees, were treated with TNF-α without or with treatment of $1,25(OH)_2D_3$ or β-catenin signaling inhibitor IWR-1-endo, at indicated times. Cell proliferation, apoptosis, and expression of SOST, β-catenin, Collagen II, and MMP13 was measured by CCK-8, flow cytometry, real-time PCR, and Western blotting, respectively. It was found that TNF-α levels were higher in synovial fluid in OA patients, compared with that in healthy controls. TNF-α treatment (5, 10, 25, 50, 75, and 100 ng/mL) markedly suppressed cell proliferation of articular chondrocytes in time- and dose-dependent manners. β-catenin signaling inhibitor IWR-endo (5 mM), as well as $1,25(OH)_2D_3$ (10 and 100 nM), significantly inhibited TNF-α-mediated cell proliferation, apoptosis, and expression of SOST, β-catenin, Collagen II, and MMP13 in articular chondrocytes. Present data suggests that $1,25(OH)_2D_3$ suppresses TNF-α-mediated cell proliferation, apoptosis, and expression of SOST, β-catenin, Collagen II, and MMP13 in rat articular chondrocytes and may be effective for prevention and treatment of OA.

Keywords: Osteoarthritis, TNF-α, $1,25(OH)_2D_3$, cell proliferation, apoptosis, β-catenin

Introduction

Osteoarthritis (OA), also known as osteoarthrosis, degenerative arthritis, and proliferative arthritis, is a chronic progressive bone and joint syndrome characterized by articular cartilage degradation. It seriously endangers the health of people [1]. Abnormal metabolism of articular chondrocytes leads to denaturation, degeneration, apoptosis, and subsequent cartilage destruction [2, 3], which are of great importance in OA development. Cytokines that regulate the balance of the catabolism and anabolism of cartilage matrix cause the destruction and degradation of cartilage matrix in OA. Interleukin-1 (IL-1) is the most important cytokine inducing articular cartilage destruction through mediating the imbalance between tissue inhibitors of metalloproteinase-1 (TIMP-1) and metalloproteinase [4]. The biological effects of TNF-α are similar to IL-1 in OA, promoting the synthesis of prostaglandin E2 (PGE2) and collagenase in fibroblasts and chondrocytes, with strong degradation effects on cartilage matrix [5, 6]. Therefore, taking effective measures to inhibit these cytokines may be a new approach for early treatment of OA.

Moreover, $1,25(OH)_2D_3$ (1, 25-dihydroxyvitamin D3) is the bioactive form of vitamin D *in vivo* involved in the metabolic regulation of bone formation and bone resorption through its roles in osteoblasts and osteoclasts [7, 8]. Bone tissue is the main target of vitamin D

and $1,25(OH)_2D_3$ plays an important role in the proliferation and differentiation of chondrocytes [9]. Furthermore, $1,25(OH)_2D_3$ can prevent undesirable hypertrophic growth plate chondrocyte differentiation during cartilage repair or regeneration [10] and inhibit Collagen II, Aggrecan, MMP9, and MMP13 expression in ATDC5 chondrocytes and articular cartilage [11, 12]. Additionally, 24R, $25(OH)_2D_3$, another vitamin D metabolite, but not $1,25(OH)_2D_3$, blocked the functions of IL-1β on rat articular chondrocytes. Protective effects against OA were, at least in part, mediated by TGF-β1 signaling pathways [13]. However, the exact roles of $1,25(OH)_2D_3$ in apoptosis and proliferation of chondrocytes require further investigation.

Wnt/β-catenin signaling pathways play an important role in maintaining the stability of bone and cartilage. β-catenin is elevated in OA patients and causes the increase of metal matrix proteases (MMPs) and aggrecanases, resulting in the destruction of cartilage [14]. Loss of sclerostin (SOST), a soluble antagonist of Wnt/β-catenin signaling, can promote OA in mice via β-catenin signaling [15]. However, the effects of β-catenin signaling on apoptosis and proliferation of chondrocytes are not fully understood. In the present study, articular chondrocytes collected from rat knees were cultured with TNF-α to establish the OA model, *in vitro*. Effects of TNF-α on cell proliferation and apoptosis of chondrocytes, as well as the underlying mechanisms of $1,25(OH)_2D_3$ inhibition of TNF-α-induced cartilage degeneration, were examined.

Materials and methods

Patient samples

Synovial fluid collected from OA patients (n=20) and healthy controls (n=20) were centrifuged at 400 × g for 20 minutes. TNF-α levels in the cell supernatant were measured by TNF-α Human ELISA Kit (KHC3011; Thermo Fisher Scientific), following manufacturer protocol.

Primary isolation and culture of articular chondrocytes from rat knee

Sprague-Dawley rats (4-week old) were sacrificed by cervical vertebra dislocation and soaked for 10 minutes in 75% alcohol. Cartilage was then obtained from the knee joint and immediately soaked in phosphate buffered solution (PBS). After cutting in a 5 mL centrifuge tube, the cartilage was digested by type II collagenase for 5 hours at 37°C and centrifuged at 400 × g for 5 minutes. After adding Dulbecco's Modified Eagle Medium (DMEM) for termination of digestion, the deposition was centrifuged at 400 × g for 5 minutes, resuspended by DMEM containing 15% fetal bovine serum (FBS), and inoculated in culture dish at 37°C in a humidified chamber with 5% CO_2. Chondrocytes at 50-60% confluence were used for immunohistochemistry assay. Afterward, they were DMEM/high glucose supplemented with 10% FBS and 1% penicillin/streptomycin and incubated with 5% CO_2 at 37°C.

Identification of chondrocytes

After being fixed by 4% formaldehyde for 30 minutes and washing with PBS for 3 minutes, a total of three times, cell slides were blocked by 3% H_2O_2 for 10 minutes and 1% bovine serum albumin (BSA) for 1 hour. They were incubated with anti-Collagen II or anti-SOX9 antibody (Abcam) at 4°C overnight. Slides were then stained with horseradish peroxidase (HRP)-labeled IgG for 25 minutes at 25°C. Subsequently, sections were stained with diaminobenzidine (DAB), counterstained with hematoxylin, and soaked in dimethylbenzene. Immunoreactive cells were counted under a microscope (× 200 magnification).

Treatment

Chondrocytes were treated with different concentrations of TNF-α (5, 10, 25, 50, 75, and 100 ng/mL) for 24, 48, and 72 hours to obtain the optimum concentration. Chondrocytes were treated with optimum concentrations of TNF-α with or without treatment of $1,25(OH)_2D_3$ (10 and 100 nM) or IWR-1-endo (5 mM) for 24 hours, observing the effects of $1,25(OH)_2D_3$ on cell proliferation, apoptosis, and protein expressions induced by TNF-α.

Cell proliferation assay

CCK-8 assay was performed using a cell proliferation and cytotoxicity assay kit (SAB, CP002). Briefly, 100 μl of cell suspension containing 3×10^3 chondrocytes was added to each well of the 96-well plates and incubated at 37°C overnight. After treatment of chondrocytes with TNF-α, $1,25(OH)_2D_3$, or IWR-1-endo, as described above, for 24, 48, and 72 hours, CCK-8 (10 μl

per well) reagent was added and reaction system was incubated for 1 hour. Cell proliferation was then evaluated using the absorbance at 450 nm.

Cell apoptosis assay

Chondrocytes were seeded in 6-well plates (5×10^5/well). They grew adherently until reaching 50% confluence. After treatment of chondrocytes with TNF-α, $1,25(OH)_2D_3$, or IWR-1-endo, as described above, for 24 hours, cell apoptosis was assessed using flow cytometry. Briefly, cells were maintained for 15 minutes in the dark at 4°C with 5 μl fluorescein isothiocyanate-labeled recombinant annexinV (Annexin V-FITC). This was followed by 5 μl propidium iodide (PI) for another 5 minutes.

Caspase-3 activity analysis

Caspase-3 Colorimetric Assay kit (KGA203; KeyGEN Biotech Co., Ltd, Nanjing, China) was used to measure Caspase-3 activity. Briefly, 3×10^6 chondrocytes were suspended by 150 μl lysis buffer containing 1.5 μl DTT, disrupted on ice for 20-60 minutes, and centrifuged at 400 × g for 5 minutes at 4°C. Next, 50 μl cell supernatant containing 100-200 μg protein was added with 50 μl 2 × reaction buffer and 5 μl Caspase-3 substrate in black for 4 hours at 37°C. Caspase-3 activity was then evaluated using the absorbance at 405 nm.

Real-time PCR

Total RNA from articular chondrocytes was extracted using the RNeasy Plus Mini Kit (Qiagen, Germany) and reversely transcribed using TaqMan reverse transcription kit (Applied Biosystems, USA). Real-time PCR was performed using the SYBR Green qRT-PCR kit (Promega, USA) on an ABI7500 system, following manufacturer instructions. Primers used in the present study are as follows: β-catenin-F, 5'-TCACGCAAGAGCAAGTAG-3' and β-catenin-R, 5'-CTGGACATTAGTGGGATGAG-3'; COL-II-F, 5'-TGGAAGAGCGGAGACTACTG-3' and COL-II-R, 5'-TGGACGTTAGCGGTGTTG-3'; SOST-F, 5'-TGATGCCACAGAAATCATCC-3' and SOST-R, 5'-ACGTCTTTGGTGTCATAAGG-3'; MMP13-F, 5'-CAGACAGCAAGAATAAAGAC-3' and MMP13-R, 5'-CAACATAAGCACAGTGTAAC-3' GAPDH-F, 5'-GGAGTCTACTGGCGTCTTCAC-3' and GAPDH-R, 5'-ATGAGCCCTTCCACGATGC-3'. Quantification of relative expression was normalized using GAPDH expression values and calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Total protein was extracted using a total protein extraction buffer (Beyotime, China). Next, 10% sodium dodecyl sulfate polyacrylamide gel was prepared to isolate the proteins. After transferring to a nitrocellulose membrane, the bands were blocked with 5% non-fat milk. Blots were incubated with primary antibodies. Secondary antibodies were diluted to appropriate concentrations and added to the protein bands, respectively. Antibodies and reagents used were as follows: β-catenin (Abcam, ab32572, 1:5000); COL-II (Abcam, ab34712, 1:5000); SOST (Abcam, ab63097, 1:1000); MMP13 (Abcam, ab39012, 1:3000); GAPDH (Cell Signaling Technology, #5174, 1:2000); HRP-labeled Goat Anti-Rabbit IgG (Beyotime, A0208, 1:1000); HRP-labeled Donkey Anti-Goat IgG (Beyotime, A0181, 1:1000); HRP-labeled Goat Anti-Mouse IgG (Beyotime, A0216, 1:1000). Results were used to visualize proteins using enhanced chemiluminescence reagents (Thermo Scientific, USA).

Statistical analysis

Data are presented as mean ± SD. Each test was repeated at least three times. Statistical analysis was conducted using one-way ANOVA with GraphPad Prism software, version 5 (GraphPad Software, USA). $P<0.05$ indicates statistical significance.

Results

TNF-α levels increased in patients with OA

TNF-α promotes the synthesis of PGE2 and collagenase in fibroblasts and chondrocytes, showing strong degradation effects on cartilage matrix [6]. Additionally, TNF-α may also induce the secretion of IL-1β in synovial cells and chondrocytes. Its activity is associated with IL-1β, which plays a synergistic role with IL-1β in the pathogenesis of OA [16, 17]. The current study found that TNF-α levels in synovial fluid in patients with OA were increased, compared with that in healthy controls (normal) ($P<0.01$; **Figure 1**), suggesting that TNF-α may be associated with the pathogenesis of OA.

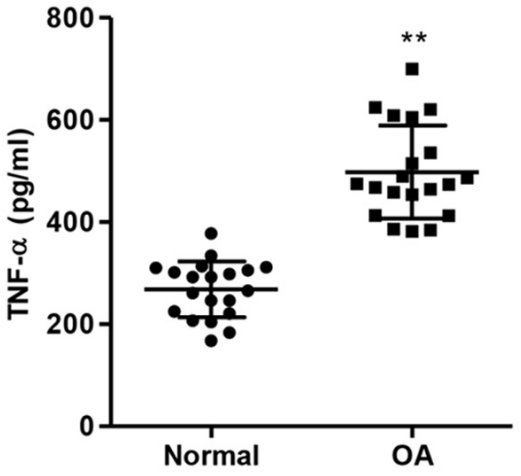

Figure 1. TNF-α levels increased in patients with OA. TNF-α levels in synovial fluid in patients with OA measured by ELISA. **P<0.01 vs. Normal.

$1,25(OH)_2D_3$ inhibits TNF-α-induced decrease in the cell proliferation of chondrocytes

To confirm the present hypothesis, articular chondrocytes from rat knees were first collected and identified by immunohistochemical staining for Collagen II and SOX9. Results demonstrated that the cells had strong protein expression of Collagen II and SOX9 (P<0.01; **Figure 2A**), indicating that cultured cells had the characteristics of chondrocytes and that chondrocytes were cultured successfully.

To investigate the effects of TNF-α on cell proliferation of chondrocytes, chondrocytes were treated with TNF-α (5, 10, 25, 50, 75, and 100 ng/mL) for 24, 48, and 72 hours. Cell proliferation was measured by CCK-8 assay. TNF-α dose-dependently suppressed cell proliferation of chondrocytes at 24, 48, and 72 hours (P<0.01; **Figure 2B**). Especially, TNF-α (5, 10, 25, 50, 75, and 100 ng/mL) treatment inhibited cell proliferation of chondrocytes by 22.4%, 30.9%, 39.9%, 51.3%, 60.0%, and 65.8% at 24 hours, compared with controls, respectively. Therefore, 50 ng/mL TNF-α was used for the following experiments. Effects of $1,25(OH)_2D_3$ on TNF-α-mediated cell proliferation of chondrocytes were examined, subsequently. It was found that $1,25(OH)_2D_3$ significantly inhibited TNF-α-mediated decreases in cell proliferation of chondrocytes in a dose-and time-dependent manner (P<0.01; **Figure 2C**). Especially, $1,25(OH)_2D_3$ (10 and 100 nM) increased cell proliferation of chondrocytes by 25.9% and 46.3% at 24 hours, compared with TNF-α treatment, respectively. IWR-1-endo (5 mM) treatment also significantly increased cell proliferation of chondrocytes by 51.7%, 90.9%, and 138.9% at 24, 48, and 72 hours, compared with TNF-α treatment, respectively (P<0.01; **Figure 2C**).

$1,25(OH)_2D_3$ inhibits TNF-α-induced increase in the cell apoptosis of chondrocytes

The function of $1,25(OH)_2D_3$ in TNF-α-mediated cell apoptosis of chondrocytes was also examined. As shown in **Figure 3A** and **3B**, 50 ng/mL TNF-α treatment significantly induced cell apoptosis of chondrocytes by 12.3-fold, compared with controls (P<0.01). Moreover, $1,25(OH)_2D_3$ (10 and 100 nM) treatment significantly inhibited TNF-α-mediated increases in cell apoptosis of chondrocytes by 27.1% and 52.5%, respectively (P<0.01). IWR-1-endo (5 mM) treatment also significantly decreased cell apoptosis of chondrocytes by 57.3%, compared with TNF-α treatment (P<0.01; **Figure 3A** and **3B**). Moreover, the Caspase-3 activity in chondrocytes with treatment of TNF-α, $1,25(OH)_2D_3$ and IWR-1-endo was also measured. It was found that 50 ng/mL TNF-α treatment significantly increased Caspase-3 activity by 2.3-fold, compared with controls (P<0.01; **Figure 3C**). Additionally, $1,25(OH)_2D_3$ (10 and 100 nM) treatment significantly inhibited TNF-α-mediated increases in Caspase-3 activity by 16.1% and 45.8%, respectively (P<0.01). IWR-1-endo (5 mM) treatment also significantly decreased Caspase-3 activity by 55.5%, compared with TNF-α treatment (P<0.01; **Figure 3C**).

$1,25(OH)_2D_3$ inhibits TNF-α-mediated expression of SOST, β-catenin, COL-II, and MMP13 in chondrocytes

Expression of SOST, β-catenin, COL-II, and MMP13 in TNF-α-induced chondrocytes was also measured by real-time PCR and Western blotting. Present data shows that 50 ng/mL TNF-α treatment significantly increased expression of β-catenin and MMP13, but decreased expression of SOST and COL-II, compared with controls (P<0.01; **Figure 4A-C**). Moreover, $1,25(OH)_2D_3$ or IWR-1-endo (5 mM) treatment significantly inhibited TNF-α-mediated expression of SOST, β-catenin, COL-II, and MMP13 in chondrocytes (P<0.01).

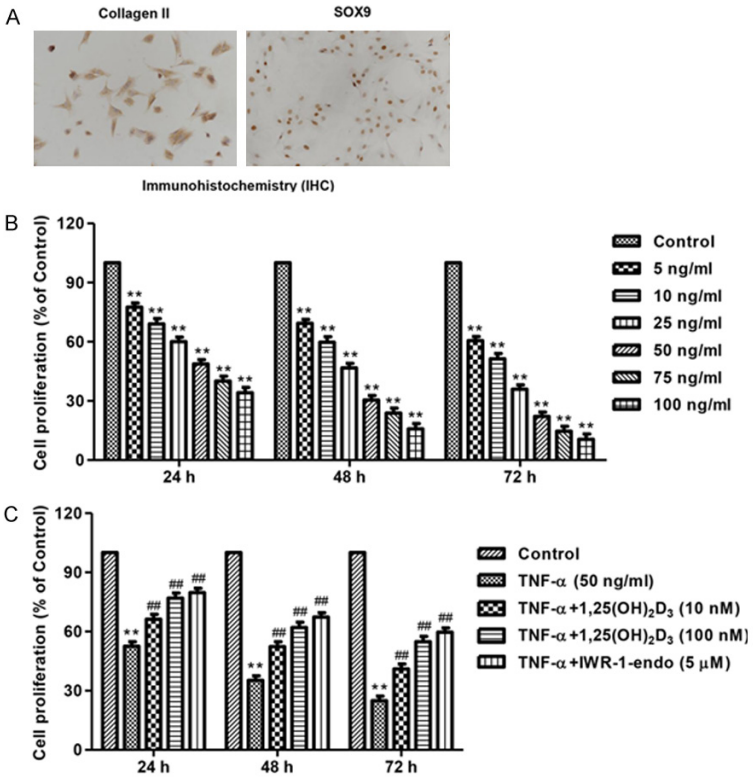

Figure 2. $1,25(OH)_2D_3$ inhibits TNF-α-induced decrease in cell proliferation of chondrocytes. A. Expression of Collagen II and SOX9 in isolated rat knee articular chondrocytes was measured by immunohistochemistry (IHC) analysis. B. Chondrocytes were treated with TNF-α (5, 10, 25, 50, 75, and 100 ng/mL) for 24, 48, and 72 hours. Cell proliferation was measured by CCK-8 analysis. C. Chondrocytes were treated with TNF-α (50 ng/mL) with or without treatment of $1,25(OH)_2D_3$ (10 and 100 nM) or IWR-1-endo (5 mM) for 24, 48, and 72 hours. Cell viability was measured by CCK-8 analysis. **P<0.01 vs. Control. ##P<0.01 vs. TNF-α.

Discussion

At present, the pathogenic factors and pathogenesis of OA are not completely clear. Previous studies have shown that the main reasons for cartilage degradation of knee OA are articular cartilage cell apoptosis, extracellular matrix degradation, and reconstruction of articular subchondral bone [18, 19]. TNF-α is a cytokine secreted by activated macrophages with many biological functions, including regulating inflammatory response, apoptosis, and antiviral response. In the present study, articular chondrocytes collected from rat knees were treated with TNF-α to establish the OA model *in vitro*. It was found that TNF-α treatment significantly inhibited cell proliferation and induced apoptosis of articular chondrocytes, which was suppressed by treatment of $1,25(OH)_2D_3$ or IWR1-endo.

Cytokines are a kind of substance with strong biological activities in the body, having many physiological functions and characteristics. IL-1, an important cytokine, promotes inflammatory effects through the production of collagenase. It also promotes the synthesis and release of PGE2 in cartilage synovial cells, thereby causing synovitis and bone resorption destruction. PGE2, in turn, further strengthens the effects of IL-1 on cartilage decomposition [20, 21]. IL-1 can enhance the activity of TNF-α and TNF-α can induce the production of IL-1. TNF-α may induce chondrocyte synovial cells secreting IL-1β and nitric oxide (NO), thereby playing a synergistic role with IL-1β and NO in the pathogenesis of OA [5, 22]. In this study, TNF-α was found to be increased in OA patients, compared with healthy controls. TNF-α treatment significantly inhibited cell proliferation and induced apoptosis of articular chondrocytes, consistent with results of a previous study [23, 24].

Chondrocyte-associated gene SOX9 is coexpressed with Collagen II during chondrogenesis in mice and in cultured chondrocytes [25]. Collagen II and SOX9 were obviously expressed in primary cultured articular chondrocytes, suggesting that the chondrocytes were successfully cultured. Osteoblast differentiation and proliferation are directly stimulated by $1,25(OH)_2D_3$ [7, 8], while the effects of $1,25(OH)_2D_3$ on proliferation and apoptosis of chondrocytes are not yet known. In the present study, $1,25(OH)_2D_3$ significantly inhibited TNF-α-mediated cell proliferation and apoptosis of chondrocytes, suggesting a protective role of $1,25(OH)_2D_3$ against TNF-α-induced cartilage degeneration in OA model *in vitro*. Type II collagen (Collagen II/COL-II) is a specific collagen of articular cartilage, distributed evenly throughout the whole cartilage, accounting for 80%-90% of total collagen. Collagen II not only par-

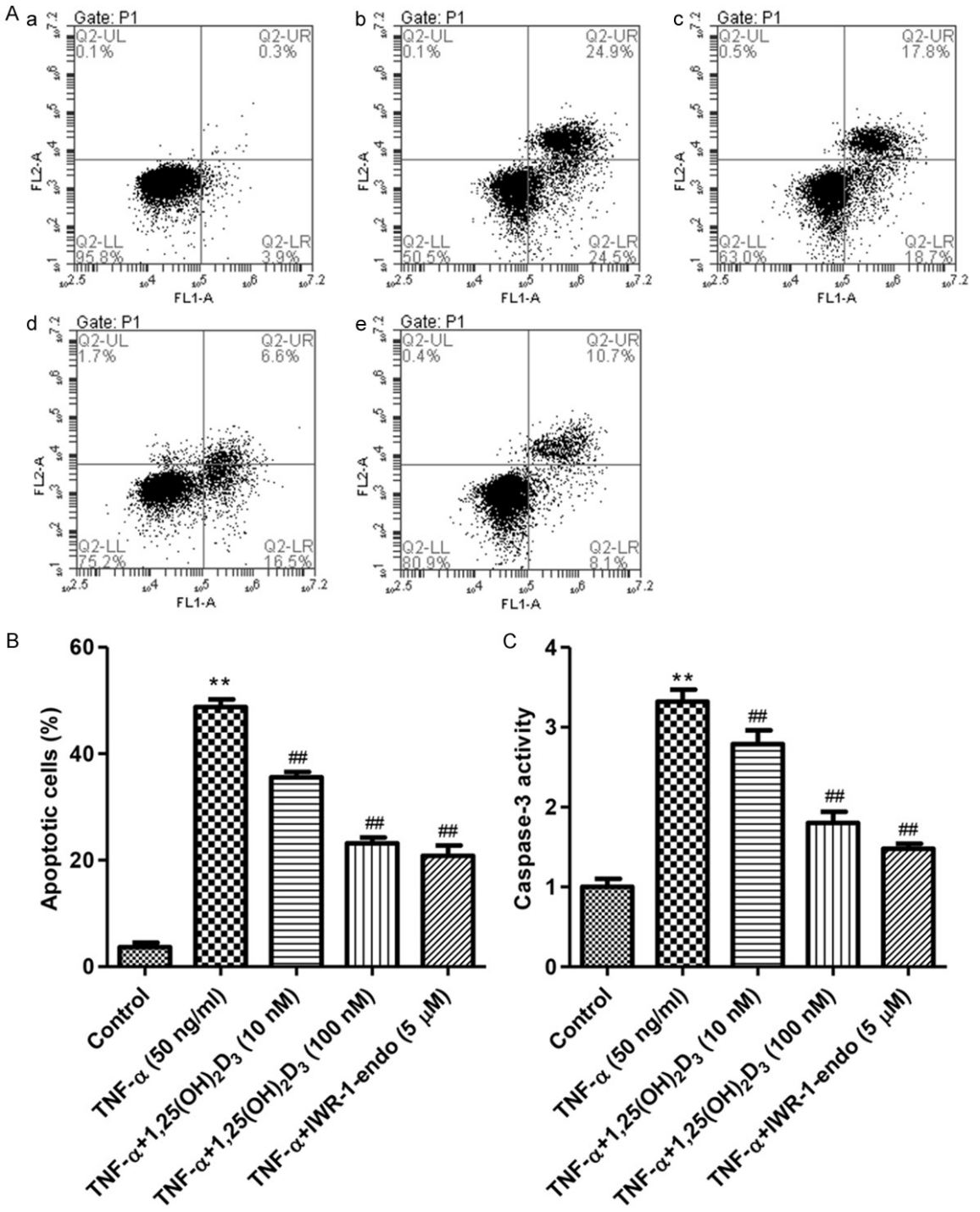

Figure 3. $1,25(OH)_2D_3$ inhibits TNF-α-induced increase in the cell apoptosis of chondrocytes. Chondrocytes were treated with TNF-α (50 ng/mL) with or without treatment of $1,25(OH)_2D_3$ (10 and 100 nM) or IWR-1-endo (5 mM) for 24 hours. Cell apoptosis was measured by flow cytometry analysis (A, B) and the Caspase-3 activity was measured by biochemical assay (C). (a) control; (b) TNF-α (50 ng/mL); (c) TNF-α+$1,25(OH)_2D_3$ (10 nM); (d) TNF-α+$1,25(OH)_2D_3$ (100 nM); (e) TNF-α+IWR-1-endo (5 mM). **P<0.01 vs. Control. ##P<0.01 vs. TNF-α.

ticipates in the regeneration and repair of articular cartilage, but also in the regulation of bone growth, development, differentiation and migration, and abnormal changes of the collagen structure. These lead to articular cartilage degeneration and dysfunction [26]. MMP13 is

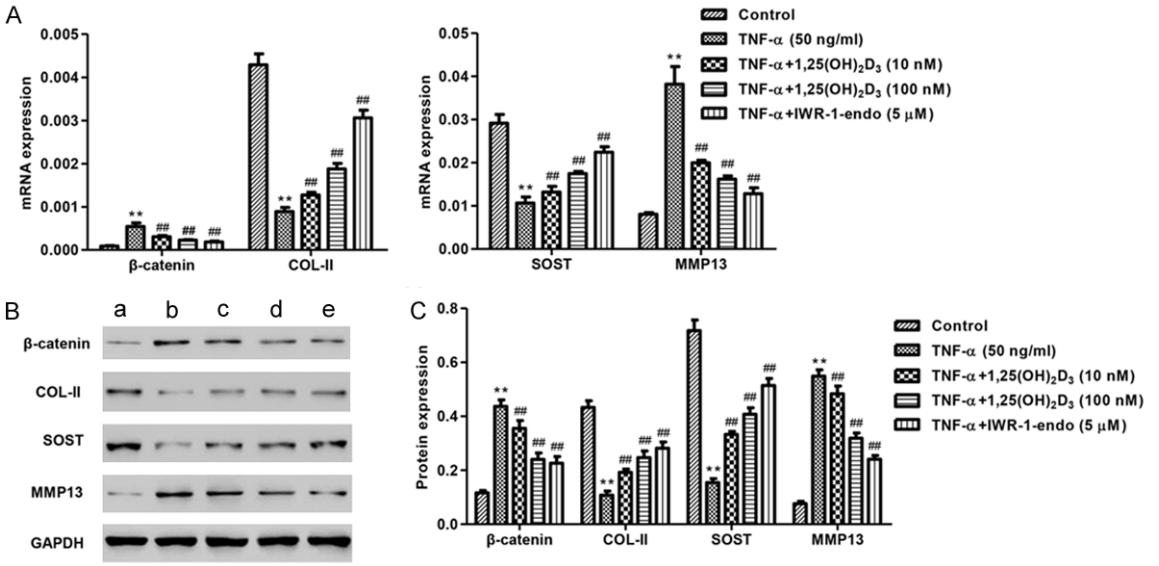

Figure 4. $1,25(OH)_2D_3$ inhibits TNF-α-mediated expression of SOST, β-catenin, Collagen II, and MMP13 in chondrocytes. Chondrocytes were treated with TNF-α (50 ng/mL) with or without treatment of $1,25(OH)_2D_3$ (10 and 100 nM) or IWR-1-endo (5 mM) for 24 hours. Expression of SOST, β-catenin, Collagen II, and MMP13 was measured by Real-time PCR (A) and Western blot assay (B, C). (a) control; (b) TNF-α (50 ng/mL); (c) TNF-α+$1,25(OH)_2D_3$ (10 nM); (d) TNF-α+$1,25(OH)_2D_3$ (100 nM); (e) TNF-α+IWR-1-endo (5 mM). **P<0.01 vs. Control. ##P<0.01 vs. TNF-α.

secreted mainly by chondrocytes and is the most effective enzyme in Collagen II degradation [27]. These studies suggest that Collagen II and MMP13 are associated with the pathogenesis of OA. Similarly, current results show that TNF-α-induced decreased Collagen II and increased MMP13 was inhibited by $1,25(OH)_2D_3$ in chondrocytes.

Wnt/β-catenin signaling has been shown to be associated with OA. Excessive increases or inhibition of the activity of β-catenin results in OA, but the mechanisms of the two are different [28, 29]. Under normal physiological conditions, β-catenin can promote chondrocyte maturation and inhibit its apoptosis, but excessive expression can accelerate the maturation of articular chondrocytes, increase protease expression, accelerate the degradation of cartilage matrix, and cause articular cartilage destruction. However, when β-catenin expression is too low to meet physiological needs, articular cartilage cells will undergo excessive apoptosis and cannot maintain and repair normal articular cartilage. These factors suggest that inappropriate expression of β-catenin is closely related to occurrence of OA. In the present study, TNF-α treatment increased β-catenin and MMP13 expression and decreased SOST and Collagen II expression in chondrocytes. This was inhibited by IWR-1-endo, a β-catenin signaling inhibitor, which also inhibited TNF-α-mediated cell proliferation and apoptosis of chondrocytes. Increased SOST expression in chondrocytes inhibited cartilage degradation in OA [1], while decreased SOST expression resulted in OA through β-catenin-dependent and-independent Wnt pathways [15].

Conclusion

In conclusion, current results indicate that TNF-α can inhibit cell proliferation and induce cell apoptosis and β-catenin signaling activation of articular chondrocytes *in vitro*. These are suppressed by treatment of $1,25(OH)_2D_3$. Therefore, $1,25(OH)_2D_3$ may serve as a novel therapeutic and β-catenin may serve as a novel therapeutic target for OA treatment.

Disclosure of conflict of interest

None.

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