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Original Article

Insulin-like growth factor binding protein 3 inhibits inflammatory response and promotes apoptosis in fibroblast-like synoviocytes of osteoarthritis

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Abstract: Synovitis plays an important role in the progression of osteoarthritis (OA). An adenovirus containing IGFBP-3 (AdIGFBP-3) was used to transform IGFBP-3 to human osteoarthritis fibroblast-like synoviocytes (FLS) in vitro. The regulating effects of IGFBP-3 in inflammation and apoptosis were investigated. AdIGFBP-3 could suppress NF-κB activation and chemokine secretion which were induced by tumor necrosis factor α (TNFα) in OA FLS and it also sensitized OA FLS to TNFα-induced apoptosis in vitro. This study suggests that the inflammatory response was reduced by the blockage of NF-κB pathway and induction of apoptotic in OA FLS by IGFBP-3. IGFBP-3 might inhibit synovitis by but it didn't have a therapeutic effect on osteoarthritis.

Keywords: Osteoarthritis, IGFBP-3, fibroblasts, inflammation, apoptosis

Introduction

Osteoarthritis (OA) is a common form of arthritis, which is the major cause of disability in elderly people. It is widely accepted that OA is characterized by articular cartilage degeneration. There is no effective drug or therapy to stop or reverse the pathophysiological progress of the disease at present, and patients often need joint replacement in the advanced stage of the disease, which compromises the quality of their daily life and brings significant financial burden to the society.

Most early studies have focused on the important role of chondrocyte apoptosis and cartilage degeneration in the progress of OA, which was considered a non-inflammatory disease before. But some recent researchers believed synovitis also exists in OA, and it could be found at all stages of the disease [1, 2]. Mononuclear cells infiltration and cytokines secretion in synovial tissues of early OA were verified by immunohistochemical staining [3, 4]. Although the inflammatory response of OA synovial tissues was found to be less pronounced than rheumatoid arthritis (RA), but there still exist one special population of immune cells (mast cell) that enriched in OA when compared to RA. Base on this, OA could be qualitatively differentiated from RA [6]. Furthermore, synovitis and effusion synovitis was associated with an increased risk of cartilage loss even patients were not diagnosed as OA [5].

Given this, synovitis seems to be one of the causes of joint degeneration in OA pathology rather than a result [6]. Unfortunately, we haven't known if the damage of joint structure could be slowed down by suppression of inflammation [7]. The role of synovitis in OA pathology needs further research apart from the chondrocyte apoptosis and extracellular matrix degradation, this study was the very one that just focuses on it.

Insulin-like growth factor binding protein 3 (IGFBP-3) is a binding protein which carries more than 70% of the circulating Insulin-like growth factor 1 (IGF-1) among 6 structurally related IGF binding proteins (IGFBPs). It has been demonstrated that IGFBP-3 had anti-inflammatory effects via inhibiting NF-κB signaling cascades pathway [8, 9, 13]. The adenovi-

ral-mediated expression of IGFBP-3 mutant with a lack of IGF binding affinity (AdmtIGFBP-3) blocked the effects of asthma by negatively regulating NF-κB signaling through IGFBP-3 receptor-mediated activation of caspases [10] and a reduction in bone destruction by reduced Chemokine (C-C motif) ligand 5 (CCL5) production in mice with collagen-induced arthritis (CIA) [11].

Moreover, IGFBP-3 is a kind of highly efficient pro-apoptotic molecule, and it is essential for TNFα-induced apoptosis in tumor cells [14], its stimulation was inhibited by IGFBP-3, not IGF-1 [12]. In addition, the interaction of up-regulated expression of IGFBP-3 and type V TGF-β causes cell growth inhibition [13, 14].

Since most early work based on the rational that RA is a chronic systemic autoimmune disease but OA is a degenerative joint disease, many studies reported the benefit of inflammation inhibited by in vivo and in vitro substances in synovial tissues of RA, but few about OA. The influence of IGFBP-3 on inflammation and cell apoptosis of fibroblast-like synoviocytes (FLS) were investigated in the present study, we try to contribute some guidance and reference for further research.

Materials and methods

Isolation and culture of human OA FLS

OA FLS were isolated from the primary synovial tissue of patients from Department of Orthopedics, Renmin Hospital of Wuhan University with whom met the diagnostic criteria of American College of Rheumatology [15] at Kellgren-Lawrence (KL) grade [16] III and IV and had undergone total joint replacement surgery or arthroscopic synovectomy. Synovial tissue was washed by phosphate-buffered saline (PBS) solution, then removed adipose and other irrelevant tissue. Synovial samples were mined into 1 mm^3 size, digested in type II collagenase (Sigma-Aldrich, MO, USA) in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, CA, USA) and shaked under 80 rpm for 1.5-2 hours at 37°C. After this, 0.25% Trypsin with EDTA (Sigma-Aldrich, MO, USA) was mixed with leftover for half an hour at 37°C after centrifuging the supernatants. The cells collected by centrifugation then were grown at 37°C in a humidified atmosphere containing 5% CO_2 in high glucose-containing DMEM supplemented with 15% fetal bovine serum (Gibco-BRL, Life Technologies, CA, USA), OA FLS were used at passages 4-8, when they consisted of a homogeneous population. The study protocol was approved by the Ethics Committees of Renmin Hospital of Wuhan University, and all patients signed the informed consent.

Immunocytochemistry

OA FLS were fixed for 15 min with 4% paraformaldehyde, washed in PBS and permeabilized with a solution containing 0.1% (for CD90/Thy-1) and 0.3% (for Vimentin) Triton X-100 and 5% BSA for 1 h. Then synoviocytes were incubated with a rabbit Vimentin (1 μg/ml) and CD90/Thy-1 (1:100) primary antibody (Abcam, Cambridge, UK) for overnight at 4°C, washed with PBS 3 times and incubated with anti-rabbit FITC and anti-rabbit CY3 conjugated secondary antibody (Abcam, Cambridge, UK) for 2 h at room temperature. The cells were then counterstained with DAPI. Images were taken using Olympus microscope and further analyzed with Image J software.

Preparation of the recombinant adenovirus

We used the AdMaxTM system (Microbix Biosystems, Ontario, Canada) to generate adenovirus as described previously [17, 18]. In brief, wild-type IGFBP-3 and IGFBP-3 mutants which provide a tool for studies directed at IGF-independent actions of IGFBP-3 were constructed in pDC316 plasmid, referred to Jiang Hong et al [21]. HEK 293 cells were used viral transfection and amplification, the adenovirus infection of OA FLS incubated with medium containing adenovirus at multiplicity of infection (MOI) of 150 per cell for 36 hours of exposure. This was kindly help by Institute of medical virology, Wuhan University School of Basic Medical Science. AdLacZ which contains empty vector was produced as a control.

Western blot analysis

OA FLS (4×10^6) were treated by 10 ng/ml TNFα serum-free solution for 24 hours after AdIGFBP-3 infection. Then were washed twice by centrifugation in PBS and pelleted at 500 g for 5 minutes. OA FLS were homogenized with protease and phosphatase inhibitors in a pro-

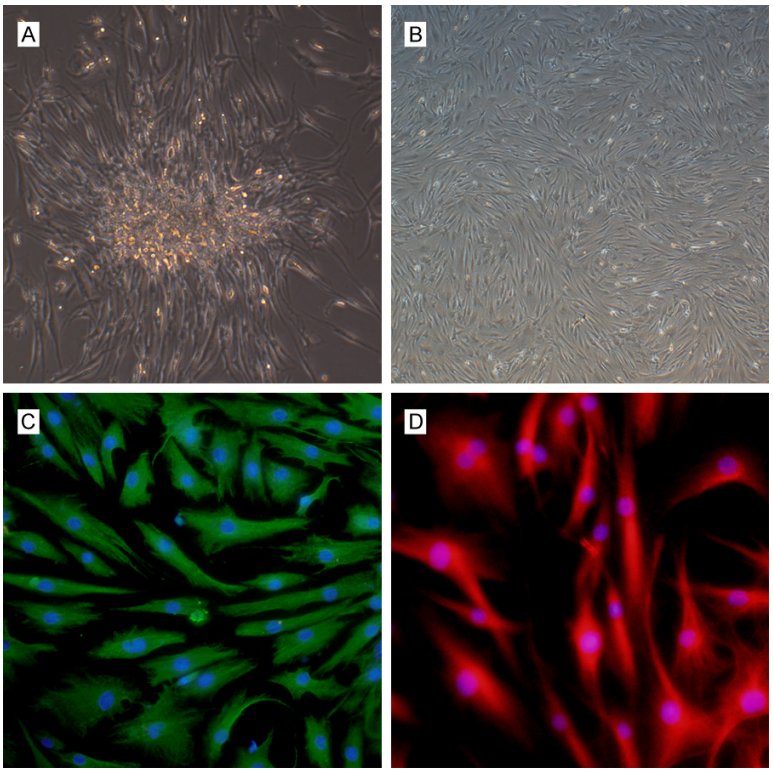

Figure 1. Fibroblast-like synoviocytes (FLS) of osteoarthritis (OA) and immunocytochemistry. Primary cells grew from synovial tissues, most of them were fusiform and polygonal (magnifiation, ×100) (A). 4th passage cells were fusiform and arranged in arc (magnifiation, ×40) (B). Positive stain of Vimentin (green) and CD90/Thy-1 (red), nucleus were blue (magnifiation, ×200) (C and D).

tein extraction solution (Sigma-Aldrich, Shanghai, China). 40 μg of protein was resolved on 10% SDS-PAGE gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). Nonspecific antibody of the membranes was blocked in 5 g/L skimmed milk for 1 h at room temperature and washed in TBS. The blot was probed with 1 μg/ml of primary antibody against IGFBP-3, NF-κB/p65, IκBα (all from Abcam, Cambridge, UK), Caspase 3, Cleaved-Caspase 3 (both from Cell Signaling Technology, Shanghai, China), bcl-2, bax (R&D systems, MN, USA). β-actin and proliferating cell nuclear antigen (PCNA) were used as loading controls for cytoplasmic and nuclear proteins, respectively.

Enzyme-linked immunosorbent assay

Serum and joint fluid were obtained from OA patients who did not have diabetes or glucose intolerance. The IGFBP-3 content, high sensitive C-reaction protein (hs-CRP) and erythrocyte sedimentation rate (ESR) level were measured by Quantikine enzyme-linked immune sorbent assay (ELISA) kits (R&D systems, MN, USA) according to the manufacturer's protocol. Following TNFα treatment, cox-2, bax, CCL5 and interleukin-1 beta (IL-1β) levels in the cell culture supernatants were determined using ELISA kits (R&D systems, MN, USA) according to the manufacturer's protocol.

Flow cytometry

Cellular apoptosis were measured by Annexin V-PE Apoptosis Analysis Kit (Sungene Biotech, Tianjin, China). OA FLS were treated by 10 ng/ml TNFα serum-free solution for 24 hours after AdIGFBP-3 infection. Then FLS (1×10^5/well) were harvested and wash with cold PBS. Suspend and centrifuge cells in binding buffer according to the manufacturer's protocol. Annexin V-PE was added into the tubes and gently vortex each tube for 10 minutes in room temperature, protected from light. Then add 7-AAD solution incubation for 5 min in room temperature, protected from light. Then these samples were analyzed by BD flow cytometry.

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical comparisons were performed using one-way ANOVA, followed by least significant difference (LSD). All statistical analyses were completed using SPSS 22 (SPSS Inc., Chicago, USA). The significance of differences between groups was determined using independent-sample t-test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Cell culture and fluorescence staining

The primary cell grew from tissues after 7 to 10 days, most of them were fusiform with few were

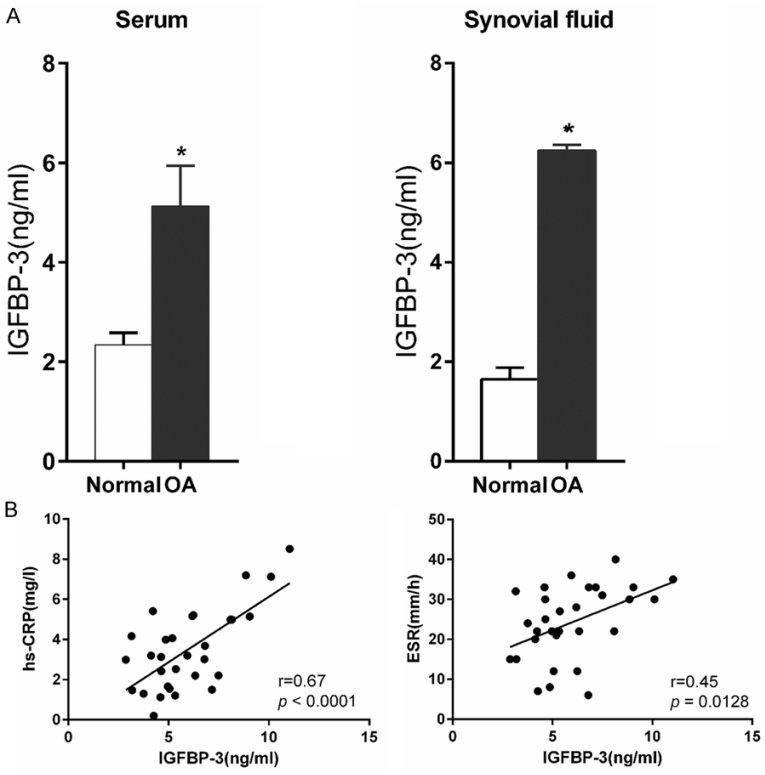

Figure 2. The IGFBP-3 level from serum and synovial fluid in OA patients (mean age 69.4 years; n = 30) were measured by enzyme-linked immune sorbent assay (ELISA) kit. Bars show the mean ± SD. *$P < 0.05$, versus normal (A). Correlations between serum concentrations of IGFBP-3 and high sensitive C-reactive protein (hs-CRP) (r = 0.67, P < 0.0001) and that of erythrocyte sedimentation rate (ESR) (r = 0.45, P = 0.0128) (B).

polygonous (**Figure 1A**, **1B**). When the synovial cells reached an 80% to 90% aggregation observed under microscope, they were passaged at a ratio of 1:2. Immunocytochemistry staining showed that Vimentin and CD90/Thy-1 protein were positive, proved that these cells were fibroblast-like synoviocytes (**Figure 1C**, **1D**).

Increased levels of IGFBP-3 from serum and joint fluid

The level of IGFBP-3 from serum and joint fluid from knee in patients with OA and were compared with normal control. The levels of IGFBP-3 from serum (5.13 ± 0.81 ng/ml) and joint fluid (6.24 ± 0.12 ng/ml) were significantly higher than normal counterparts (2.34 ± 0.24 ng/ml, 1.65 ± 0.23 ng/ml, respectively, P < 0.05) (**Figure 2A**). In the same manner, the levels of IGFBP-3 from serum (mean hs-CRP level 3.48 ± 2.02 mg/l, mean ESR level 24.20 ± 9.20 mm/h) were measured. Then the level of serum IGFBP-3 and hs-CRP or ESR of every patient was taken by correlation analysis. When FLS were stimulated with TNFα, level of IGFBP-3 increased. The results indicated that the serum level of hs-CRP and ESR had a significant positive correlation with IGFBP-3 level in patients with OA (**Figure 2B**).

Overexpression of adenovirus-mediated IGFBP-3 inhibit inflammatory response in OA FLS

The adenovirus expressing IGFBP-3 under the control of a cytomegalovirus promoter (AdmtIGFBP-3) was used to determine the inhibition of NF-κB activity of IGFBP-3 on OA FLS, following the exposure of TNFα, a potent pro-inflammatory cytokines. After stimulated with TNFα of RA-FLS, the nuclear levels of p65 increased, the expression of IL-1β, MMP-1, and CCL5, which were NF-κB target genes, were increased (**Figure 3A-C**). It has been reported that IκBα, but not IκBβ, was the major participant in cytokine-induced NF-κB activation in FLS [19]. After TNFα treatment, there was an increase in pro-inflammatory protein but decrease in IκBα (**Figure 3D**).

These findings clearly demonstrated that the IGFBP-3 inhibited the TNFα-induced nuclear translocation of p65, IκBα degradation and NF-κB target genes' secretion in OA FLS, suggesting the ligand-independent effect of IGFBP-3 on the suppression of NF-κB target genes.

Overexpression of adenovirus-mediated IGFBP-3 promoted apoptosis of OA FLS

Given that the pro-apoptotic effects on FLS from RA and cartilage cells, we were curious IGFBP-3 would regulate apoptosis of OA FLS or not. To do this, OA FLS were stimulated by TNFα for 24 hours, the level of Caspase 3, Cleaved-Caspase 3, bcl-2, bax and CCL5 were

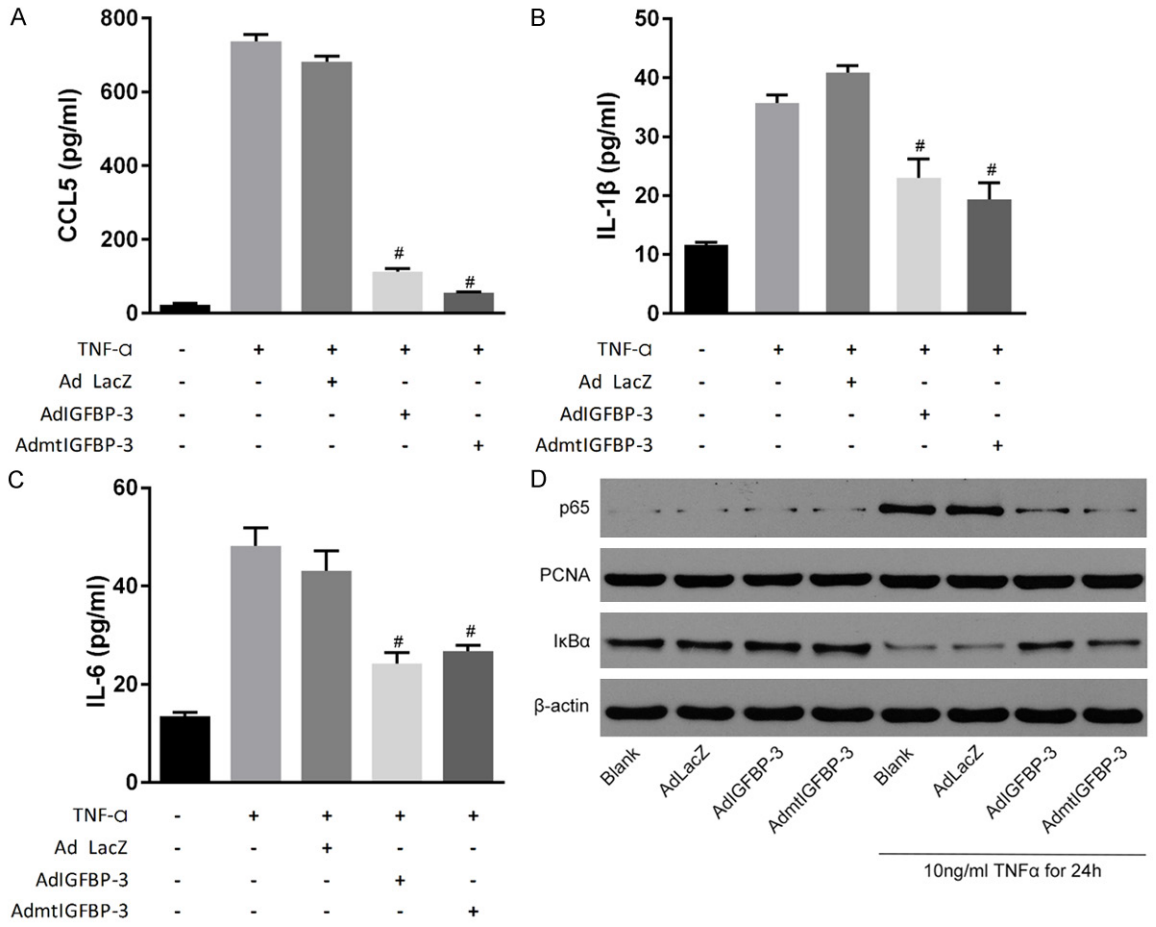

Figure 3. Inhibition of tumor necrosis factor α (TNFα)-induced production of chemokines and cytokines in osteoarthritis (OA) fibroblast-like synoviocytes (FLS) by insulin-like growth factor binding protein 3 (IGFBP-3) overexpression. OA FLS were infected with either AdLacZ, AdIGFBP-3, or AdmtIGFBP-3 at 150 multiplicities of infection (MOI) for 36 hours and then treated with TNFα (10 ng/ml) for 24 hours. Levels of CCL5 (A), interleukin-1β (IL-1β) (B) and interleukin-6 (C) in the cell-free culture supernatants, evaluated by enzyme-linked immune sorbent assay (ELISA) kit. Cytoplasmic degradation of IκBα and nuclear translocation of p65 and p50 subunits, determined by Western blot. β-actin and proliferating cell nuclear antigen (PCNA) were used as loading controls for cytoplasmic and nuclear proteins, respectively (D). In (A-C), bars show the mean ± SD from 3 independent experiments. #P < 0.05 versus AdLacZ.

measured by Western blot. Compared with the AdLacZ group, Bcl-2 was lower in AdIGFBP-3 group, while caspase 3/cleaved caspase 3 and Bax were higher, indicated that there was a pro-apoptotic effect by IGFBP-3 in OA FLS (**Figure 4A**).

The ratios of apoptosis were observed by flow cytometry (**Figure 4B**, **4C**), Compared with negative control and AdLacZ group, IGFBP-3 appeared to significantly induce apoptosis in OA FLS which treated with TNFα for 24 hours. In the negative control, the frequency of apoptotic FLS was only 1.92 ± 0.26%, which was significantly lower (all P < 0.05) than measurements in AdLacZ group (3.75 ± 0.63%), AdIGFBP-3 group (9.13 ± 0.52%) and AdmtIGFBP-3 group (10.50 ± 0.20%), suggesting that IGFBP-3 induced apoptosis in OA FLS.

Discussion

In this study, we investigated whether negative regulation of NF-κB by IGFBP-3 would decrease the inflammatory responses in OA FLS. The inflammatory responses in TNFα-stimulated FLS were inhibited by overexpression of IGFBP-3 by autocrine and led to the inhibition of NF-κB pathway. In addition, IGFBP-3 inhibited proliferation and induced apoptosis of OA FLS.

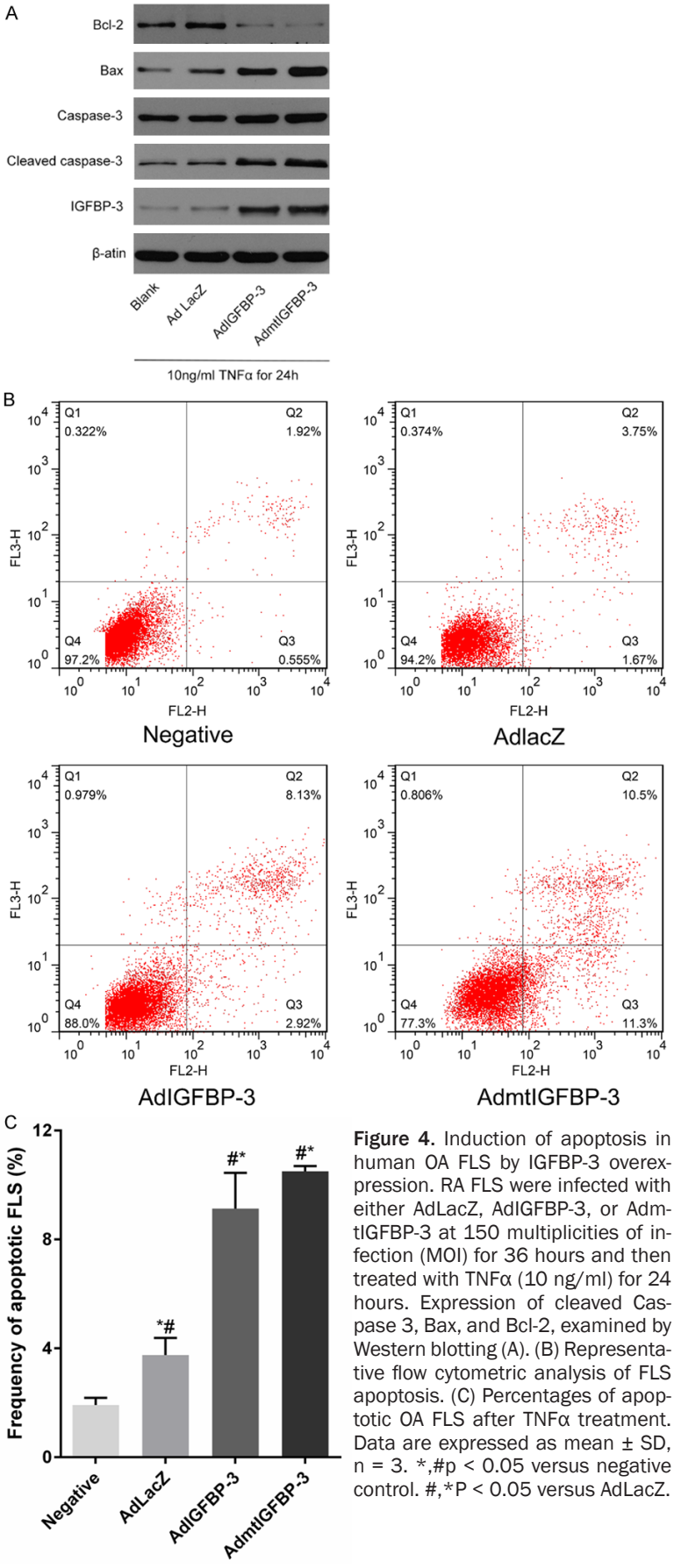

Figure 4. Induction of apoptosis in human OA FLS by IGFBP-3 overexpression. RA FLS were infected with either AdLacZ, AdIGFBP-3, or AdmtIGFBP-3 at 150 multiplicities of infection (MOI) for 36 hours and then treated with TNFα (10 ng/ml) for 24 hours. Expression of cleaved Caspase 3, Bax, and Bcl-2, examined by Western blotting (A). (B) Representative flow cytometric analysis of FLS apoptosis. (C) Percentages of apoptotic OA FLS after TNFα treatment. Data are expressed as mean ± SD, n = 3. *,#p < 0.05 versus negative control. #,*P < 0.05 versus AdLacZ.

Previous studies have shown that IGFBP-3 levels in serum and synovial fluid of OA patients increased in different degrees, Iwanaga et al [20] and Eviatar et al [21] found that up-regulation of IGFBP-3 was observed from OA cartilage tissue and explant culture. Furthermore, there were more IGFBP-3 secreted by chondrocytes over the area of the surface than deep zones of the cartilage and enhanced expression of IGFBPs was positively correlated with the histologic score for cartilage lesions [20]. This explains the elevation of IGFBP-3 from synovial fluid, but the elevation from serum may indicate that OA is not just a disease of joint; it may be a systemic disease, together with the positive correlation between IGFBP-3 and hs-CRP and ESR, which should be considered in OA therapy.

Elevated hs-CRP levels reflect synovial inflammation and were associated with severity of pain in OA patients [22, 23], which indicate that synovitis is an important risk factors, and it is highly correlated with disease progression. He et al [24] reported that hs-CRP level was above normal hs-CRP in Kellgren-Lawrence (KL) grade II, and there was no difference in hs-CRP level among KL grades II, III, and IV, which indicates severe OA. It seems that the level of ESR and hs-CRP that detect patients with OA can be used to diagnose OA, but Hanada et al [25] believed that we can't do so here because of the large overlap of ESR and hs-CRP values between the OA and non-OA groups. We reckon in the early progression from KL grade I to II, hs-CRP level might be a predictor of OA at least.

IGFBP-3 not only takes the capability of binding serum free IGF but also exhibits distinct IGF receptor-independent actions. Since Oh et al [26] found specific binding of IGFBP-3 to cell surface proteins in Hs578T human breast cancer cells. The intracellular trafficking of IGFBP-3 from which contain sequences with the potential for nuclear localization [27]. Apart from its receptor-independent functions, IGFBP-3 also could interfere with NF-κB signaling cascades, supported by Lee et al [10] whose work declared that IGFBP-3 could prevent asthma induction without IGF-1. These effects of IGFBP-3 seemed to be IGF-I independent, because our result and Lee et al [10] research indicated that AdIGFBP-3^{GGG} showed similar results. In this study, we investigated whether negative regulation of NF-κB by AdIGFBP-3 and AdmtIGFBP-3 would affect inflammatory responses in OA FLS. Cytokines like p65, IL-1β were clearly inhibited by IGFBP-3, as well as IκBα degradation. These indicated that overexpression of IGFBP-3 inhibited the NF-κB pathway, which in turn, inhibited inflammatory responses in TNFα-stimulated FLS.

Interestingly, we observed that there was big change of OD value of IL-1β in some groups, with large standard deviation. Benito et al [1] reported similar results. Compared with the results of Smith et al [28] and Myers et al [29], these contradictory findings might be explained that synovial tissues in the present study were taken only from advanced stage OA patients.

For the reason why synovial membrane becomes inflamed, the widely accepted hypothesis was that inflammatory mediators were produced by synovial cells when cartilage fragments fall into the articular cavity then contact the synovium, which were considered as foreign bodies. These inflammatory mediators led to the cartilage degradation, synovial angiogenesis and the production of MMPs. It seems that synovitis perpetuates the cartilage degradation in OA [30]. In early OA, overexpression of inflammatory mediators had been observed [1, 31], suppression of inflammation response by IGFBP-3 might be a beneficial treatment of early OA.

We previously reported that IGFBP-3 could be imported into chondrocyte and exerted IGF-1 independent effect, meditating a translocation of Nur77 from nucleus to mitochondria and inducing chondrocyte apoptosis as a result [32]. Our study suggested that IGFBP-3 influenced the expression of Bcl-2, Bax, and Caspase 3, which caused FLS apoptosis.

Based on the anti-inflammatory and pro-apoptosis effects of IGFBP-3, it could inhibit synovial inflammation in OA, which may be one of the directions for the therapy of OA. But IGFBP-3 can promote the apoptosis of chondrocytes, which is a disadvantage for OA, in particular, chondrocytes has an important position in the progress of OA.

To summarize, the present study demonstrates that IGFBP-3 may be a potential therapy target for OA, for it reducing synovial inflammation by inactivating the NF-κB pathway and its downstream signals. But its pro-apoptotic effect on cartilage cells and inhibition on synovial membrane may produce an adverse effect, more likely to have adverse effects. Further investigation will be done to find a balance.

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Disclosure of conflict of interest

None.

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