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Effects of TNF-α in rheumatoid arthritis via attenuating α1 (I) collagen promoter

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Abstract. – OBJECTIVE: To explore the role of TNF-α in the peripheral blood of patients with rheumatoid arthritis (RA) and its underlying mechanism.

PATIENTS AND METHODS: 32 patients diagnosed with RA in our hospital from July 2016 to March 2017 were selected in the experimental group. Meanwhile, 32 normal healthy people were selected in the healthy control group and 21 patients with other autoimmune diseases in the same period were selected in the disease control group. Serum samples of the subjects in the experimental group and the control group were collected. The content of serum tumor necrosis factor-α (TNF-α) was detected by enzyme-linked immunosorbent assay (ELISA), and the correlation between TNF-α and RA activity was analyzed. We then constructed rat RA model. The effect of different doses of TNF-α on the RA progression was evaluated by measuring the foot paw thickness of both hind limbs of rats. Fibroblast-like synoviocytes (FLS) were treated with different concentrations of TNF-α cytokine in vitro. Cell counting kit-8 (CCK-8) assay was carried out to detect the cell viability after TNF-α treatment. Serum levels of VEGF (vascular endothelial growth factor) and hydroxyproline were detected. Moreover, the α1 (I) collagen overexpression recombinant was constructed and transfected into MH7A cells. The activation of α1 (I) collagen promoter was reflected by the CAT reporter gene activity.

RESULTS: ELISA results showed higher content of TNF-α in the peripheral blood of the experimental group than that of the control group. In the RA rat model, the foot paw thickness of the hind limbs was increased with the increase of TNF-α concentration. CCK-8 and colony formation assay demonstrated that the proliferation of MH7A cells was elevated after TNF-α treatment. Higher levels of VEGF and IL-6 secreted by FLS and decreased collagen synthesis ability of MH7A cells were found after TNF-α treatment. Transfection of the α1 (I) collagen overexpression recombinant in MH7A cells led to the reduced activity of CAT after TNF-α treatment, suggesting that the activation of α1 (I) collagen promoter was inhibited.

CONCLUSIONS: TNF-α participates in RA by inhibiting the activation of the promoter of α1 (I) collagen, as well as enhancing the secretion of VEGF and IL-6 in MH7A cells.

Key Words:
Rheumatoid arthritis, TNF-α, α1 (I) collagen.

Introduction

Rheumatoid arthritis (RA), as a chronic autoimmune disease, is manifested as persistent synovitis and systemic articular cartilage and bone destruction, which eventually leads to joint malformation and dysfunction[1,2]. RA is also considered to be one of the main causes for human disability[3]. Fibroblast-like synoviocytes (FLS) exert a key role in the pathological progression of RA[4]. Functionally, FLS exhibit tumor-like characteristics, including abnormal proliferation, anti-apoptosis and release of pro-inflammatory cytokines[5]. Meanwhile, FLS are involved in the pathogenesis of RA, which are capable of regulating the immune response and inflammation by secreting inflammatory mediators, pro-inflammatory cytokines and anti-inflammatory cytokines, leading to the hyperplasia of synovial tissues. At the same time, it may participate in the degradation of extracellular matrix, bone and joint destruction, and promotion of angiogenesis by secreting matrix metalloproteinases (MMPs)[6]. Accumulating evidence has shown that inhibited proliferation of FLS can effectively prevent joint injury and maximize the improvement of disease condition,

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so as to improve life quality of RA patients. Therefore, our study mainly explored the cellular functions of FLS.

Many investigations[7] have indicated that tumor necrosis factor-α (TNF-α) is the key cytokine of synovial inflammatory response in RA, which play a crucial role in the pathogenesis of RA. TNF-α almost participates in all aspects of the inflammatory reaction of RA, such as the aggregation and activation of inflammatory cells, and FLS proliferation[8]. Additionally, TNF-α is overexpressed in the synovia, synovium and cartilaginous pannus of RA patients.

TNF-α is mainly produced by synovial macrophages. It can stimulate FLS proliferation, and secrete effector molecules including IL-6, macrophage colony stimulatory factor (GM-CSF), chemokines, metalloproteinases and prostaglandins[9]. However, few studies have explored the exact role of TNF-α in the collagen synthesis of FLS. In this study, the aim was to detect the concentration of TNF-α in peripheral blood of RA patients, and to explore the effect of TNF-α on FLS proliferation and collagen synthesis.

Patients and Methods

Patients

A total of 85 patients who received treatment in our hospital from July 2016 to March 2017 were selected in this experiment. Enrolled subjects were assigned into three groups, including the experimental group (32 RA patients), healthy control group (32 normal healthy people) and disease control group (21 patients with other autoimmune diseases). There were 11 males and 21 females in the experimental group, with the mean age of 45.7±15.1 years. 13 males and 19 females were included in the healthy control group with the mean age of 44.9±10.2 years. There were 8 males and 13 females in the disease control group with the mean age of 42.6±18.4 years. Among 21 patients with other autoimmune diseases, 9 cases had systemic lupus erythematous, 5 had osteoarthritis, 3 had gout, 1 had polymyositis/dermatomyositis, 1 had ankylosing spondylitis, 1 had systemic sclerosis and 1 had primary desiccation syndrome. No significant differences were found in age and gender among the three groups ($p>0.05$). The inclusion criteria were applied as follows: (1) all RA patients were in accordance with the RA diagnostic criteria and active standard of the American College of Rheumatology (ACR) in 1987[10]. Diagnosis of other diseases was in accordance with the corresponding diagnostic criteria; (2) all subjects had normal liver and kidney function; (3) informed consent was obtained in every subject. The exclusion criteria were applied in those combined with severe organ disease or malignant tumor, pregnancies or lactation patients and received hormone therapy or slow acting drug treatment within four weeks. This study was approved by the Ethics Committee of Affiliated Hospital of Taishan Medical University. Signed written informed consents were obtained from all participants before the study.

Construction of the Rat RA Model

Emulsified bovine type II collagen was used to induce the rat RA model after adaptive feeding for 3days. The specific procedures were as follows: A certain amount of bovine type II collagen was dissolved in 0.1 mol/L glacial acetic acid, so that the final dose was adjusted to 2 mg/mL. The solution was then placed overnight at 4°C. Equal volume of glacial acetic acid containing bovine type II collagen and complete Freund›s adjuvant were mixed in ice bath. The mixture was grinded until complete emulsification. The concentration of the collagen was 1 mg/mL.

Except for rats in the control group, other rats were anaesthetized by intraperitoneal injection of pentobarbital sodium and sterilized with 75% alcohol. 1mL of prepared bovine collagen type II collagen solution was administrated into both sides of the spine and tail by subcutaneous point injection. After 1 week, 0.5 ml of solution was injected again to strengthen the immune response. No special treatment was performed in rats from the control group.

After successful construction of rat RA model, rats in the TNF-α-1, TNF-α-2 and TNF-α-3 groups were injected with 1mLof TNF-α with the concentrations of 1, 5 and 10 μg/mL, respectively. TNF-α injection was performed twice a 1 week, for a total of 3 weeks. No treatment was performed in rats from the control group and RA group.

Foot thickness of rats was detected in each group. Briefly, an electronic vernier caliper (Anyi Instrument, Guilin, China) was used to measure the palmar thickness of hind limbs in rats before model construction and 1 week after TNF-α injection. Each measurement was repeated for 3 times. Rats were sacrificed after the final measurement of palmar thickness. Knee joint was exposed and the synovial tissue was completely stripped. Rat

tumor necrosis factor (TNF-α) quantitative detection kit (KGI Biological Science and Technology Development Co. Ltd, Nanjing, China) was used for detecting the content of TNF-α in the synovial tissue of rats.

Cell Culture

The synovial fibroblasts cell line MH7A was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (HyClone, South Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in a 5% CO_2 incubator at 37°C, and the culture medium was replaced every 2-3 days. Cells were passaged when the cell confluence was up to 80-90%.

Cell Transfection

The cells in the logarithmic growth phase were seeded into the 6-well plates. When the cell confluence was about 60%, cells were transfected according to the instructions of Entranster-R4000 (EngreenBiosystem, Beijing, China). Briefly, 500 μL of serum-free suspension containing 4 μL of Entranster-R4000 and 2.68 μg of recombinant plasmid was prepared. 500 μL of cell suspension and 1.5 mL of 1640 culture medium (Gibco, Grand Island, NY, USA) were added for incubation. Meanwhile, equal amounts of Entranster-R4000 and pcDNA-control were added to the control group. The culture medium was replaced after transfection for 6 h.

Western Blot

The transfected cells were lysed with the cell lysate containing protease inhibitor (RIPA lysate) (Beyotime, Shanghai, China), and the total protein was extracted. The concentration of each protein sample was determined by the NanoDrop 2000 spectrophotometer (Hitachi, Tokyo, Japan). Subsequently, 5×loading buffer was added for protein denaturation. Briefly, total protein was separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) under denaturing conditions and transferred to PVDF (polyvinylidene fluoride) membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk, followed by the incubation of specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight. Membranes were then incubated with the secondary antibody at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence method.

Cell Apoptosis Assay

The cell suspension was collected into marked flow tubes, and digested with Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin. After centrifugation, the cells were washed with PBS (phosphate buffer saline) twice. We then added 200 μL of binding buffer containing calcium ions (Invitrogen, Carlsbad, CA, USA), 10 μL of Annexin V-FITC (Fluorescein Isothiocyanate) fluorescent probe and 5 μL of propidium iodide (PI) to the cell precipitation, followed by incubation without light for 5 min. Subsequently, cell cycle was analyzed by flow cytometry (Partec AG, Arlesheim, Switzerland) through FL1 and FL3 dual channels.

Construction of the α1 (I) Collagen Overexpression Plasmid

According to the sequence of pCOLH 5.3, regulation sequence of the 5'-UTR region of human α1 (I) collagen gene with different lengths and recombinant pCOLH of CAT reporter gene that referred to human α1 (I) collagen gene upstream -2483-+42 bp sequence were constructed. Primers for amplification of recombinant fragments and the specific construction process were designed according to previous studies[11]. Primer sequences are as follows: forward: 5' -ATGTCTACGCGTACATCTTCAGCC TGGGCAC-3', reverse: 5′-ATAGTACTCGAGCGTGCCTCCTGCTCCGAC-3′.

Statistical Analysis

We used statistical product and service solutions (SPSS22.0, Armonk, NY, USA) Software for all statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x} \pm s$). The *t*-test was used for comparing differences between the two groups. $p < 0.05$ was considered statistically significant.

Results

TNF-α Content in the Peripheral Blood of RA Patients was Associated with RA Activity

Totally 85 patients who received treatment in our hospital from July 2016 to March 2017 were selected. ELISA results showed that TNF-α con-

tent in the peripheral blood of the experimental group (132.76 ± 50.98 pg/mL) was remarkably higher than that of the control group (50.32 ± 32.09 pg/mL) (Figure 1A, $p < 0.05$). Moreover, TNF-α expression was positively correlated with RA activity ($r = 0.6908$, $p < 0.05$, Figure 1B-1C).

TNF-Đ Increased the Degree of Joint Swelling in the RA Rat Model

Totally 30 male Wistar rats were selected and randomly assigned into the control group, RA group, TNF-α-1 group, TNF-α-2 group and TNF-α-3 group, with 6 rats in each group. Except for rats of the control group, the rat RA model was established by injecting emulsified bovine type II collagen with Freund's complete adjuvant. Foot paw thickness of hind limbs, and TNF-α content in the serum and synovial tissue of rats were measured before and after TNF-α injection, respectively. The data showed that after TNF-α injection for 1 week, higher foot paw thickness of hind limbs (Figure 2A) and TNF-α content in the synovial tissue (Figure 2B) were found in the TNF-α group than those of the RA group ($p < 0.05$). Additionally, foot paw thickness of the hind limbs increased with the increase of TNF-α concentration, indicating that TNF-α in peripheral blood aggravates RA development in rats.

TNF-α Promoted FLS Proliferation and Secretion of VEGF and IL-6 in Vitro

Our study found that the proliferation of MH7A cells was enhanced with the increased concentration of TNF-α in a certain range (0-10 ng/mL) (Figure 3A). CCK-8 and colony formation results indicated a higher proliferation rate of MH7A cells in the TNF-α group than that of the control group (Figure 3B-3C). It is reported that inflammatory factors are involved in FLS proliferation. Hence, we speculated whether TNF-α promotes the proliferation of MH7A cells *via* secreting inflammatory factors. Western blot results showed an elevated expression of VEGF in MH7A cells treated with TNF-α (Figure 3D). To explore the effect of TNF-α on the adjacent cells, we then performed ELISA to detect expressions of VEGF and IL-6 in cell supernatants. Of note, the data demonstrated that expressions of VEGF and IL-6 were elevated in cell supernatant supplemented with 10 ng/mL TNF-α, suggesting that TNF-α could promote FLS proliferation *via* secreting VEGF, IL-6 and other RA related cytokines.

TNF-α Inhibited Collagen Synthesis by Inhibiting the Activation of Human α1 (I) Collagen Promoter in Vitro

Flow cytometry showed that TNF-αremarkably inhibited apoptosis of MH7A cells (Figure 4A). Previous study indicated that TNF-α could reduce the level of type I collagen gene activated by TGF-β1. In this study, content of type I collagen in MH7A cells remarkably increased in the culture medium supplemented with TNF-α (Figure 4B). Meanwhile, we detected the level of hydroxyproline (HYP), an indicator of collagen degradation in cell supernatant. ELISA results showed that TNF-α remarkably promoted degradation of type I collagen (Figure 4C). The vector pCA T3-Enhancer (4293bp) is a plasmid containing the SV 40 enhancer and the chloramphenicol acetyltransferase (CAT) reporter gene without promoter. To explore whether TNF-α inhibited collagen synthesis through the α1 (I) collagen promoter, we constructed the α1 (I) collagen

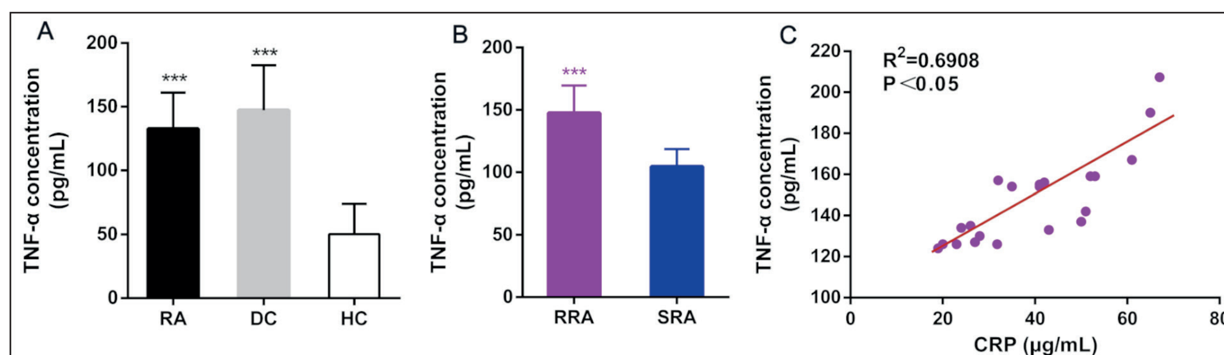

Figure 1. The correlation between TNF-α in peripheral blood and RA. ***A,*** The level of TNF-α in the peripheral blood of the experimental group and the disease control group was significantly higher than that of the normal healthy control group. ***B,*** The level of TNF-α in the peripheral blood of active RA patients was significantly higher than that of non-active RA patients. ***C,*** TNF-α in peripheral blood was positively correlated with CRP ($r = 0.6908$, $p < 0.05$).

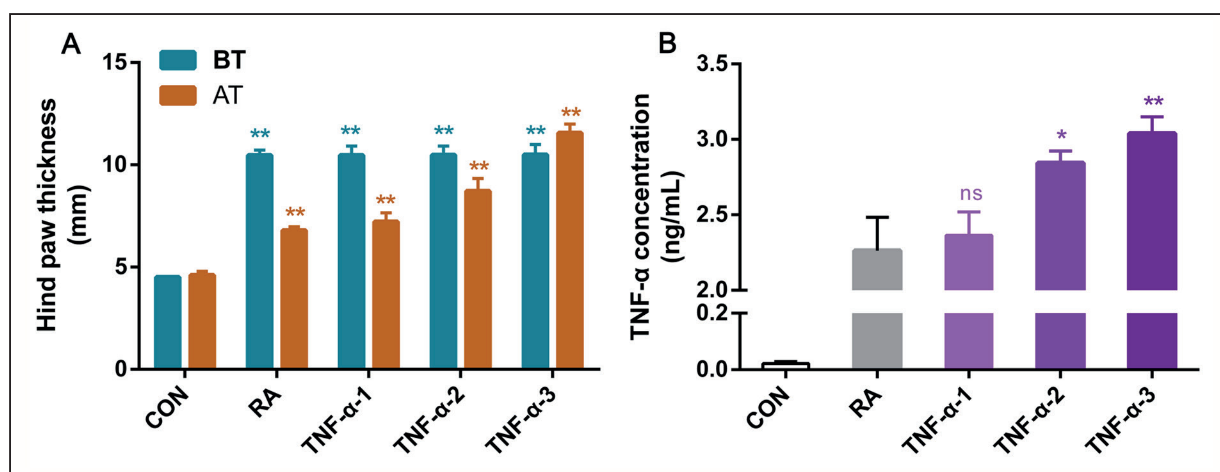

Figure 2. Effect of TNF-α on the disease process of the rat RA model. ***A,*** Foot paw thickness of the TNF-α group was significantly increased when compared with that of the control group. Moreover, the degree of foot swelling increased with the increase of TNF-α concentration. ***B,*** In the TNF-α group, the content of TNF-α in synovial membrane increased with the increase of the injected TNF-α concentration.

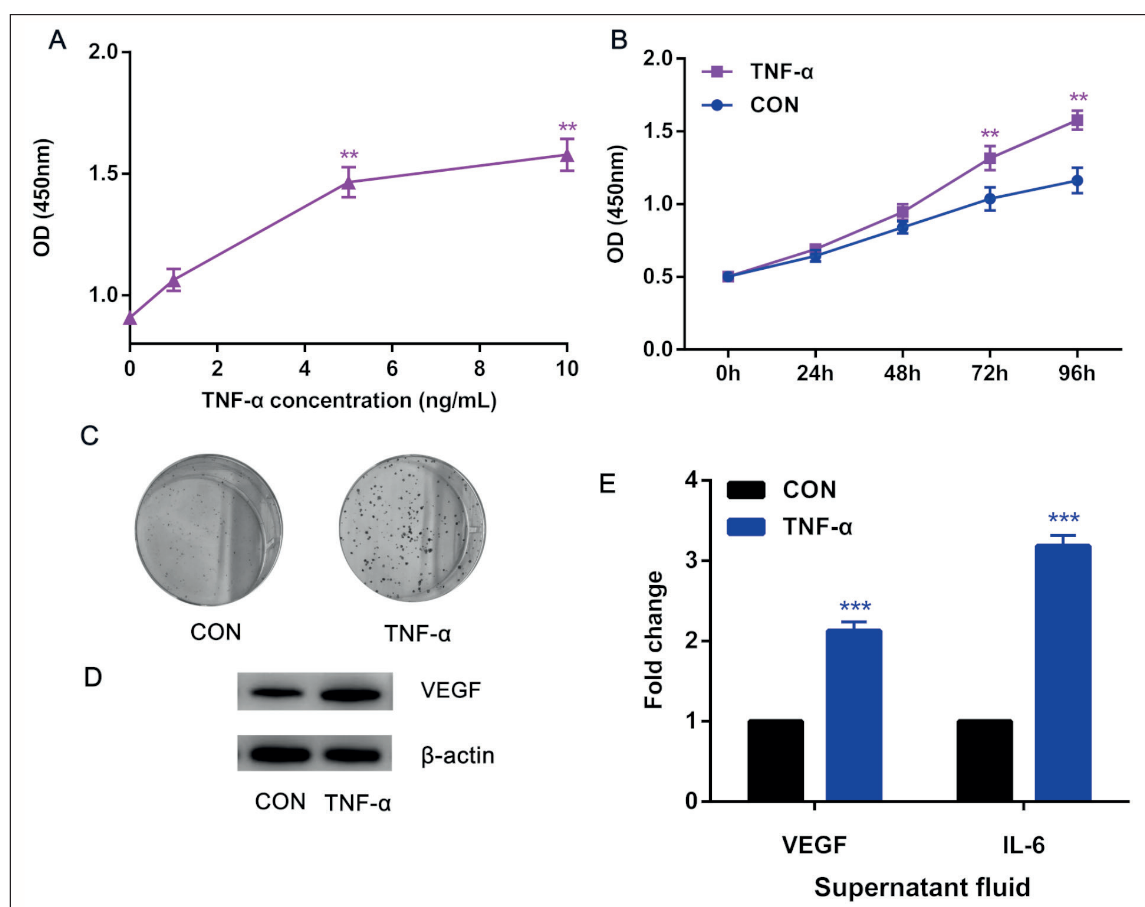

Figure 3. Effect of TNF-α on FLS proliferation and secretion of VEGF and IL-6 *in vitro*. ***A,*** The proliferation of MH7A cells increased with the TNF-α concentration. ***B,*** CCK-8 results showed that the proliferation rate of MH7A cells increased significantly in the culture medium supplemented with TNF-α. ***C,*** Colony formation showed that the ability of MH7A cells to form clones was significantly enhanced in the culture medium supplemented with TNF-α. ***D,*** Western blot results showed that the expression of VEGF in MH7A increased significantly in the culture medium supplemented with TNF-α. ***E,*** ELISA results showed that the secretion of VEGF and IL-6 in the supernatant of MH7A increased significantly when cultured with 10 ng/mL TNF-α.

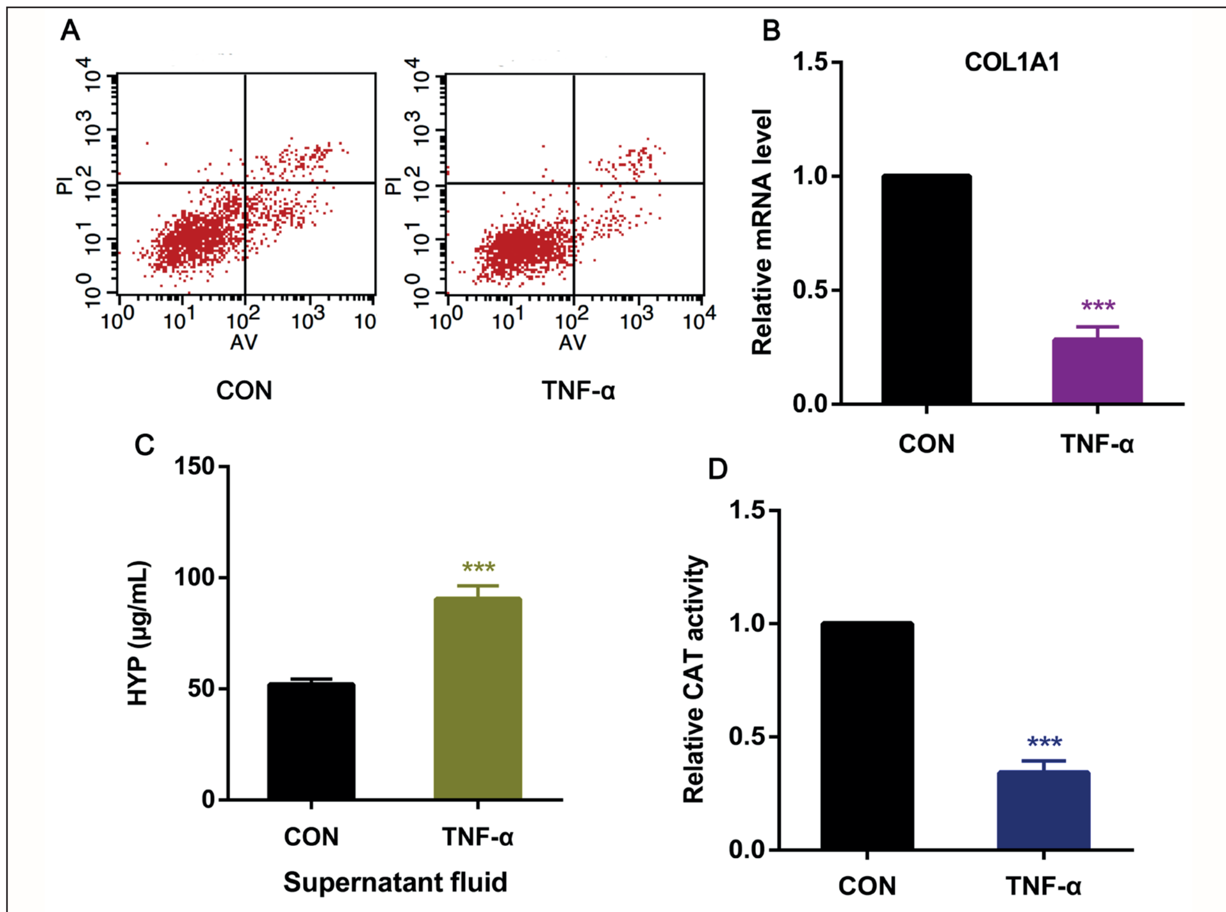

Figure 4. Effect of TNF-α on collagen synthesis *in vitro*. ***A,*** Flow cytometry showed that the apoptosis of MH7A cells decreased significantly in the culture medium supplemented with TNF-α. ***B,*** Western blot results showed that the expression of type I collagen in MH7A cells increased significantly in the culture medium supplemented with TNF-α. ***C,*** ELISA results showed that TNF-α enhanced the degradation of collagen in MH7A cells. ***D,*** CAT activity of the TNF-α group decreased significantly compared with the control group.

overexpression vector by the pCA T3-Enhancer. In the present study, decreased CAT activity of the TNF-α group was found after transfection of the overexpression plasmid in MH7A cells (Figure 4D), suggesting that TNF-α suppressed the activation of the α1 (I) collagen promoter.

Discussion

RA is a chronic autoimmune disease with high incidence and disability rate[11-13]. The basic pathological manifestations of RA include synovitis, proliferation of synovial cells and erosion of cartilage and bone[14]. Macrophage-like synovial cells (MLs) and Fibroblast-like synoviocytes (FLS) are distributed in the synovial membrane. FLS account for about 2/3 of synovial cells, which are the major source of inflammatory cytokines and matrix degrading enzymes[15]. Cytokines, such as IL-1, IL-6 and IL-8, can stimulate synovial cells to produce inflammatory response, release MMPs, promote connective tissue degradation and pannus formation, eventually leading to RA[16]. Thus, FLS exert a crucial role in RA pathogenesis. Thickening of the synovial membrane is one of the most significant pathological features of RA. The synovial lining layer consists of 15 layer cells, which is mainly caused by the increased FLS number. There are many inflammatory cells that are infiltrated in the sub-synovial layer. In some RA patients, FLS proliferation is more significant than that of inflammatory cells, which may be associated with cell apoptosis and aging[17].

Various inflammatory cytokines are released,

which eventually result in structural damage and dysfunction of joints in RA patients. Among these inflammatory cytokines, TNF-α is the most crucial one in RA occurrence[18]. TNF-α is mainly produced by monocytes, macrophages and T cells[19]. The main role of TNF-α in RA pathogenesis is to induce inflammatory cytokines, including IL-1β and IL-6, eventually aggravating the inflammatory response[20]. Meanwhile, TNF-α can induce monocytes and macrophages to produce and release chemokines, and recruit lymphocytes to the inflammation site from blood vessels. Overexpression of TNF-α can induce the secretion of protein hydrolase and metalloproteinase in macrophages and fibroblasts, respectively[21,22]. This may cause tissue destruction of the articular cartilage and subchondral bone, as well as osteoclast reabsorption.

In this study, the results of ELISA showed that TNF-α content in peripheral blood of RA patients was higher than that of patients in the control group. In the RA rat model, the disease condition was aggravated with the increase of TNF-α concentration. Moreover, we found that TNF-α promoted proliferative ability of MH7A cells, which also stimulated the secretion of IL-6 and VEGF *in vitro*. On the other hand, TNF-α led to an obvious decrease of collagen synthesis in the MH7A cells. Additionally, after transfection of the α1 (I) collagen recombinant to MH7A cells, we found that TNF-α could inhibit the activation of the promoter of α1 (I) collagen.

Conclusions

We detected that TNF-α participates in RA by inhibiting the activation of the promoter of α1 (I) collagen, as well as enhancing the secretion of VEGF and IL-6 in MH7A cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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