Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by joint inflammation and bone destruction. The pro-inflammatory mediator nitric oxide (NO), a catalytic product of inducible nitric oxide synthase (iNOS), plays a crucial role in the progression of RA.1,2 The levels of NO and iNOS are significantly elevated in RA patients and in the synovial tissues of animals with inflammatory arthritis.3–6 The NO synthase inhibitor NC-monomethyl-L-arginine can significantly inhibit the incidence of arthritis in animal models, demonstrating the vital role of iNOS and NO in the incidence and progression of arthritis as well as the destruction of tissues.7
Kruppel-like factor 2 (KLF2), a zinc finger transcription factor with anti-inflammatory and anti-oxidation functions, can regulate the anti-coagulation function of endothelial cells and control vascular tone. However, few reports exist on the pathophysiological significance of KLF2 in chronic and inflammatory diseases such as RA. KLF2 was recently reported to regulate monocyte differentiation and to play a role in methylated-BSA and interleukin (IL)-1β-induced arthritis.

In this study, we examined whether KLF2 is expressed in mice with collagen-induced arthritis (CIA) and assessed the correlation of KLF2 expression with the severity of arthritis and the presence of inflammatory mediators. We also investigated the effect of KLF2 silencing on the expression of iNOS in RA synoviocytes.

Materials and methods

Animals

Eight-week old male DBA/1 mice were supplied by the medical animal center in Zhejiang province and were maintained in a pathology-free animal facility. This study was approved by the Institutional Ethics Committee of Taizhou University. To calculate the sample size selected, a statistical test of two independent samples (Fisher exact test, two-tail) was used. Six to eight mice would be required to each group to achieve a power of 80% with a significance level of 0.05. We performed the sample size calculation according to the method from the reference.

Preparation of the collagen-induced arthritis mice model

Twenty milligrams of bovine type II collagen (CII) was dissolved in 10 mL of 0.1 M acetic acid at a concentration of 2 mg/mL, then mixed with complete Freund’s adjuvant (CFA) at a ratio of 1:1 for a final concentration of 1 mg/mL. The mixture was injected into the tail veins of the mice, with a booster injection administered 7 days later to establish the CIA disease model. Similar mice without CII injection were used as a control.

Scoring and grouping of arthritis index

The severity of arthritis in the limbs of the mice was scored on the 28th day after the first injection and twice a day thereafter. The scoring standard used for arthritis in the limbs was as follows: 0: not red or swollen; 1: red and swollen little toe joints; 2: red and swollen metatarsophalangeal joints; 3: red and swollen joints below the ankle; and 4: red and swollen foot including ankle joints. The scores for each joint were summed to obtain the total score for each mouse, with a maximum possible score of 12. The CIA mice were classified into mild (scores of 1–3), moderate (scores of 4–7), or severe groups (scores > 8) 56 days after the injection of CII.

Histopathological observation and scoring of joints

The mice were decapitated 56 days after CII injection and their joints immediately retrieved. The joints were fixed in 10% methanol after peeling, followed by decalcification in EDTA and dehydration in an ethanol gradient. After paraffin embedding, sectioning, and hematoxylin and eosin (HE) staining, histological assessment was performed. The results were compared with those for the unstimulated control mice.

Determination of inflammatory mediators in joint fluid

The inflamed joints of CIA mice were cut open after 56 days of stimulation and immersed in 300 μL Dulbecco’s modified eagle medium (DMEM) (Life Technologies) for 24h. The supernatant was collected and the concentrations of tumor necrosis factor (TNF)-α and IL-6 were quantified using an ELISA kit, according to the manufacturer’s instructions (eBioscience). The nitrate reductase method was adopted to measure the NO concentration in the supernatant, following the kit manufacturer’s instructions (Nanjin Jiancheng Bioengineering Research Institute, China).

Cultivation of synovial cells

The RA fibroblast-like synoviocyte cell line, MH7A, was supplied by Jennio Biological Technology (Guangzhou, China). The cells were cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM) (Life Technologies) with high glucose-containing 10% fetal bovine serum (Life Technologies) and incubated at 37°C in a 5% CO₂
atmosphere. Cells were passaged when 90% confluent using 0.25% trypsin then seeded onto 6-well plates at a density of $5 \times 10^5$ per well.

**KLF2 short interfering RNA (siRNA) transfection**

The KLF2 siRNA (sense: 5'-CCUGGCGCUG-CACUGAAATT-3') and scrambled control siRNA (ScRNA) (sense: 5'-UUUCUCCGAAC-GUGUCACGUTT-3') were synthesized by Genepharma Inc (Shanghai, China). Twenty nanomolar KLF2 siRNA or scrambled siRNA was transfected into the cells according to the instructions for the Lipofectamine RNAiMax kit (Invitrogen). After 48 h, the transfected cells were collected and the silencing effect was measured using quantitative polymerase chain reaction (PCR) and western blot (WB) analysis.

**Western blot analysis**

Tissue homogenate or cells were lysed by incubation with RIPA lysis buffer on ice for 30 min, after which the protein supernatant was collected. The buffer was mixed with 20–30 μg supernatant and the proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The samples were transferred to polyvinylidene fluoride membranes (Millipore, MA, USA) using the wet transfer method (380 mA, 50–60 min). The membranes were immersed in 5% skimmed milk and sealed using 0.2% Tween-20 surfactant at room temperature for 2 h, followed by the addition of a primary antibody (mouse anti-iNOS polyclonal, 1:1000, Abcam; mouse anti-KLF2 polyclonal, 1:2000, Abcam; rabbit anti-GAPDH monoclonal, 1:3000, Santa Cruz) overnight at 4°C. After rinsing, secondary antibodies conjugated with horseradish peroxidase (Santa Cruz) were added and incubated for 1h. Complexes of the supernatant protein and the membrane antibodies were detected using enhanced chemiluminescence (Millipore) and imaged with a gel imaging system. Grayscale analysis was performed and GAPDH was used as the endogenous control.

**Real-time PCR analysis**

After treatment, total RNA was extracted from the cells using Trizol reagent (Invitrogen) and reverse transcribed into first strand cDNA using a RevertAid first strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). Quantitative PCR was performed using an ABI Prism 7700 system (Applied Biosystems, Foster City, CA, USA) under the following conditions: 10 μL SYBR green PCR master mix, 100 ng cDNA, and 0.2 M sense and antisense primers with a total volume of 20 μL. GAPDH was used as the internal reference. Quantitative analysis of KLF2 and iNOS was achieved using the comparative Ct method ($2^{-\Delta\Delta C_t}$). The primers used were as follows: GAPDH, forward: 5'–CGC TGA GTG GTG GGA GTC-3'; GAPDH, reverse: 5’–GCT GAT GAT CTT GAG GCT GTT GTC-3'; iNOS, forward: 5’–TCA CCA GGA GAT GCT GAA CTAC-3'; iNOS, reverse: 5’–GAG CAC AGC TTT GAC CAA GAC-3'; KLF2, forward: 5’–GCA CAG ATG GCA CTG GAA TGG-3'; KLF2, reverse: 5’–GAA GAC CTA CAC CAA GAG TTC GCA-3'.

**Statistical processing**

The experimental data were processed using SPSS version 18.9 software and expressed as mean ± SD. Comparisons between two groups were analyzed using a t test, while one-way analysis of variance (ANOVA) was used to compare multiple groups. $P < 0.05$ indicated differences that were statistically significant.

**Results**

**Histopathological changes and the production of pro-inflammatory mediators in mice with CIA**

DBA/1 mice were treated with bovine CII mixed with CFA, with a booster injection administered 7 days later. The severity of arthritis in normal and CIA mice was scored. No redness or swelling was observed in the joints of normal mice during the experiment, whereas CIA mice had swollen joints after the 28th day with increasing disease severity as the experiment progressed, evidenced by the increase in the arthritis scores (data not shown). On day 56 after the first immunization, the CIA mice were classified into mild, moderate, and severe groups according to the arthritis scores. Each group of mice was sacrificed and the joint tissues and paws were harvested. Arthritis severity was determined based on synovial inflammation and bone destruction. As shown in Figure 1(a) and (b), inflammatory cell...
infiltration (mainly lymphocytes) was observed in the synovial tissues of the CIA mice, with clear indications of cartilage and intraosseous destruction. More severe synovial inflammation and bone destruction was observed in joint tissues with higher arthritis scores.

We also investigated the production of TNF-α, IL-6, and NO in mice with CIA. As shown in Figure 1(c)–(e), the TNF-α, IL-6, and NO levels in the knee joints of the CIA mice were significantly higher than the levels in the normal mice. As the disease progressed, the expression of the inflammatory mediators also increased. However, no significant difference was observed in the level of NO in the serum of the CIA mice compared with that of the normal mice (data not shown).

Expression of iNOS and KLF2 in synovial tissue, heart, and kidneys of CIA mice

We next investigated the expression of KLF2 and iNOS in the synovial tissues of CIA mice using western blotting. As shown in Figure 2(a), the expression of KLF2 and iNOS in the synovial tissues of the CIA mice clearly increased compared with the expression in the normal group. The expression of KLF2 and iNOS increased with the progression of inflammation. We also investigated the expression of KLF2 and iNOS in the hearts and kidneys of CIA mice using western blotting. As shown in Figure 2(b) and (c), the expression of KLF2 and iNOS in the heart and kidneys also increased compared with the expression in normal mice. Furthermore, the expression of KLF2 and iNOS increased with an increase in the arthritis score.

The effect of KLF2 in the expression of iNOS in RA synoviocytes

We investigated whether KLF2 and iNOS expression can be induced by the presence of TNF-α with for various durations in RA synoviocytes. TNF-α stimulated the expression of KLF2 and iNOS in a
time-dependent manner. The maximum expression of KLF2 and iNOS mRNA and protein was observed at 2 h and 16 h after incubation with TNF-α (data not shown).

To investigate whether KLF2 regulates iNOS expression in RA synoviocytes, KLF2 siRNA sequences and negative control ScRNA sequences were transfected into the RA synoviocyte cell line, MH7A, and KLF2 knockdown was determined through quantitative PCR and western blotting (Figure 3(a) and (b)). The optimum inhibitory effect was observed at a concentration of 20 μM. KLF2 knockdown not only inhibited the mRNA and protein expression of iNOS under basic conditions, it also inhibited the expression despite stimulation with TNF-α (Figure 3(c) and (e)).

**Discussion**

KLF2 is associated with acute and chronic inflammation through the regulation of inflammatory mediators. Here, we demonstrated that the increasing expression of KLF2 correlated with the severity of arthritis and high levels of inflammatory mediators TNF-α, IL-6, and NO in CIA mice. The expression of KLF2 and iNOS was up-regulated not only in synovial tissues, but also in the heart and kidney tissues of CIA mice, suggesting that KLF2 regulates the expression of iNOS in these mice.

A number of investigations have demonstrated that iNOS and its catalytic product NO are involved in RA pathology. iNOS is highly expressed in the synovial tissues and cartilage of RA patients, with macrophages of the synovial lining and fibroblasts being the principal source. Sakurai et al. showed that NO levels strongly correlated with iNOS positive synovial cells. Thus, it is likely that infiltrating macrophages and synovial fibroblasts are a significant source of NO in RA synovial synovium. In vitro, the presence of inflammatory mediators such as TNF-α, IL-1, and IFN-γ induces iNOS expression, resulting in high levels of NO in human macrophages and RA synoviocytes. These findings suggest that inhibition of the iNOS pathway indirectly regulates the expression of NO, presenting a novel strategy for RA therapy. In this study, we demonstrated that KLF2 silencing inhibits the mRNA and protein expression of iNOS induced by TNF-α in RA synovial fibroblasts, suggesting that KLF2 is involved in the activation of synovial fibroblasts and the expression of inflammatory mediators. However, the detailed mechanisms of iNOS regulated by KLF2 in RA synoviocytes remain unclear. In addition, due to the important role of macrophages in RA inflammation, whether iNOS can be regulated by KLF2 in macrophages remains to be elucidated.
Conclusion

In summary, this study suggests the expression of KLF2 correlates with synovial inflammation in CIA mice and KLF2 may be a crucial transcription factor in RA synovial inflammation by regulating the iNOS expression in RA synoviocytes.

Animal welfare

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

Ethical approval for this study was obtained from Institutional Ethics Committee of Taizhou University (TZYXY2019-201).

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