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Establishing method and expression of related proteins of collagen-induced arthritis model in DBA/1 mice

Xingda Cai[1,a], Keyu Zeng[1,b], Weijian Zhang[1,c], Dingsheng Zha[1,2,d,*]

[1]Department of Orthopedics, The First Affiliated Hospital of Jinan University, Guangzhou, 510623, China
[2]Department of Orthopedics, The Affiliated Shunde Hospital of Jinan University, Foshan, 528305, China
[a]caixingda678@163.com, [b]zengkeyu0228@163.com, [c]1021543634@qq.com, [d]zdsuser@126.com
**Corresponding author*

Abstract: *The establishment of collagen-induced arthritis (CIA) model in DBA/1 mice, along with the evaluation of relevant indicators, lays a solid foundation for studying the pathogenesis of rheumatoid arthritis. Twelve DBA/1 mice was randomly assigned to either a control or a model group, consisting of six mice per group. Mice within the model group were vaccinated with bovine typeIIcollagen (CII) to elicit collagen-induced arthritis (CIA). The changes in body weight, paw swelling degree, and arthritis index score were observed and recorded. Hematoxylin-eosin (HE) staining was used to observe the pathological changes of ankle joint. Immunohistochemistry was used to detect the expression of PI3K/AKT/mTOR signaling pathway proteins, S100A4 and VEGF. Compared with the control group, the mice in the model group exhibited a marked reduction in body weight ($P<0.05$), with apparent paw swelling and a notably elevated arthritis index score ($P<0.01$). Histological analysis of the ankle joints in the model group showed an increase in synovial tissue, a substantial infiltration of inflammatory cells, pannus formation, degeneration and necrosis of articular cartilage, subchondral bone destruction, as well as a narrowed joint space. Additionally, the abundance of proteins within the PI3K/AKT/mTOR signaling pathway, S100A4 and VEGF in the ankle joint tissue of the model group were significantly upregulated ($P<0.01$). The method of inducing CIA in DBA/1 mice utilizing bovine CIIis reliable, boasting a high incidence rate of CIA. The body weight, paw swelling, arthritis index, and ankle joint pathology of the mice can serve as crucial indicators for evaluating the CIA model. The elevated expression of PI3K, AKT, mTOR, S100A4, and VEGF in the synovial membrane of CIA mice indicates that these factors may potentially serve as targets for the treatment of RA.*

Keywords: *Collagen-induced arthritis; PI3K/AKT/mTOR signaling pathway; Evaluation indicators; Pathogenesis*

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease that predominantly targets the joints[1]. Clinically, it is characterized by joint pain and swelling, with an insidious and progressive onset. Initially, it may affect one or a few joints but often progresses to symmetric polyarthritis. The small joints of the wrists, hands, and feet are typically the most affected, larger joints can also be involved[2]. Currently, there is no definitive cure for rheumatoid arthritis, and conventional therapies are often associated with suboptimal outcomes. Patients frequently experience relapses within a short period after treatment[3]. Extensive research has been conducted to elucidate the pathogenesis of RA, but the precise biological mechanisms are still not fully understood. Recently, many researchers have explored the role of the phosphatidylinositol-3 kinase (PI3K)/protein serine-threonine kinase (Akt)/mammalian target of rapamycin (mTOR) signaling pathway in RA, achieving notable results[4]. Recent studies have demonstrated that S100 calcium-binding protein A4 (S100A4) is highly expressed in the synovial tissue of knee joints in patients with RA. S100A4 enhances the secretion of vascular endothelial growth factor (VEGF) by rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs), which in turn promotes the formation of synovial blood vessels and contributes to the development and progression of pannus[5-6].

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The collagen-induced arthritis (CIA) model serves as an ideal animal experimental model for investigating the etiology and pathogenesis of rheumatoid arthritis, as well as for evaluating the therapeutic effects of drugs. In 1977, Trentham and colleagues innovatively developed the collagen-induced arthritis mouse model to facilitate more in-depth research into rheumatoid arthritis[7]. Type II collagen (CII) derived from rats, cattle, and chickens can be used to induce an immune response that leads to arthritis. The experimental animals typically selected for this model are rodents and primates. This study utilized the immunization of DBA/1 mice with bovine type II collagen to establish CIA model. The CIA model was evaluated by assessing changes in mice body weight, arthritis index, pathological alterations in ankle joints, and immunohistochemical analysis. Furthermore, the expression of the PI3K/AKT/mTOR signaling pathway, S100A4, and VEGF in the synovial tissue of CIA mice joints was investigated. These findings provide valuable theoretical and experimental insights into the pathogenesis of RA and aid in the identification of potential therapeutic targets.

2. Materials

Animals: Male DBA/1 mice, aged 7-8 weeks and weighing 20±2 g, were obtained in SPF grade from Huafukang Biotechnology Co., Ltd., Beijing, with certificate number SYXK (Beijing 2020-0004). The mice were housed in the Animal Experiment Center of Jinan University under controlled conditions of 22-24°C temperature, 50-60% humidity, and a 12-hour light-dark cycle. They had free access to water and were provided with a standard diet. Following a 1-week acclimatization period, the CIA model was induced. Throughout the experiment, animals were treated humanely and in compliance with ethical guidelines for animal care.

Reagents: Complete Freund's Adjuvant and Immuni-zation Grade Bovine TypeIICollagen were purchased from Chondrex, Inc. (USA). AKT, mTOR, and VEGF antibodies were purchased from Shenyang Wanlei Biotechnology, Inc. (China). The S100A4 antibody were purchased from Abcam, Inc. (UK). The PI3K antibody were purchased from Signalway Antibody, Inc. (USA). The horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Shanghai Biyuntian, Inc. (China).

3. Experimental methods

3.1 Group grouping, model establishment and disposal method

Twelve male DBA/1 mice were randomly assigned to a control group and a model group using a random number table, with six mice in each group. Bovine type II collagen was completely dissolved in 0.05 mol/L acetic acid and incubated overnight at 4°C. On ice, complete Freund's adjuvant was mixed with bovine type II collagen in equal volumes and stirred to achieve emulsification. On day 1, the emulsion was subcutaneously injected at the proximal 1 cm of the mouse tail, with each mouse receiving 100 μL. The control group received an equivalent volume of saline. On day 21, a second booster immunization was administered using the same method, with each mouse receiving 100 μL.

3.2 Body weight measurement and paw lesion observation of CIA mice

The body weight of mice in each group was measured on day 0 prior to the induction of inflammation and at 7, 14, 21, 28, 35, and 42 days post-induction. Additionally, the development of paw lesions in the mice was monitored.

3.3 Arthritis score

The arthritis index (AI) score for mice assesses the degree of redness and swelling in each paw on a scale from 0 to 4. A score of 0 indicates no joint redness or swelling; 1 point is assigned for redness without swelling in the paw or toe joints, mild joint redness and swelling, or obvious redness and swelling in the finger or toe joints; 2 points for moderate redness and swelling in the ankle or wrist joints; 3 points for significant redness and swelling throughout the entire paw; and 4 points for severe redness and swelling or deformity in multiple joints of the paw. The total AI score is the sum of the scores from the front and hind limbs, with a maximum possible score of 16.

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3.4 Ankle joint pathology examination

Forty-two days post-model induction, mice were euthanized, and their ankle joints were collected. The joints were fixed in formaldehyde solution, followed by decalcification, dehydration, clearing, and embedding to create paraffin blocks. Sections were subsequently stained with HE and examined under a microscope to assess pathological alterations in the joint tissues of the mice.

3.5 Immunohistochemical detection of the expression of PI3K/AKT/mTOR signaling pathway proteins, S100A4, and VEGF

Mice joint tissues paraffin sections were deparaffinized, rehydrated, and repaired. They were then blocked with goat serum and incubated overnight at 4°C with primary antibodies against PI3K, AKT, mTOR, S100A4, and VEGF (diluted 1:100). Secondary antibodies (diluted 1:50) were added and incubated at 37°C for 1 hour. DAB was used for color development, followed by counterstaining with hematoxylin. The sections were then dehydrated and mounted. Cellular protein staining was observed, and immunohistochemistry results were analyzed using Image-Pro Plus 6.0 software to assess the integrated optical density (IOD) and positive expression area. The average optical density was calculated as the IOD divided by the positive expression area.

3.6 Statistical methods

Statistical analyses were conducted using SPSS 22.0 software, and all graphs were generated using GraphPad Prism 9. Data are expressed as mean ± standard deviation (mean±SD). For group comparisons, one-way analysis of variance (ANOVA) was employed, and results were considered statistically significant when $P < 0.05$.

4. Results

4.1 Body weight changes of the CIA mice

Following the initial immunization, mouse body weight was measured every 7 days to monitor changes. Mice in the model group showed decreased appetite, weight loss, and reduced activity 7 days after modeling, which were significantly different from those in the control group ($P < 0.05$), Figure 1.

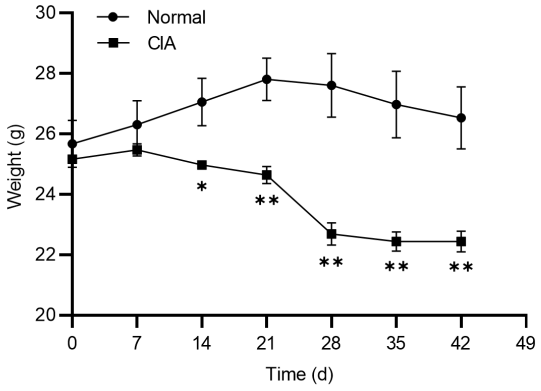

*Figure 1: Body weight changes of the CIA mice. Mean±SD. * P<0.05, ** P<0.01 vs normal.*

4.2 Status of paw lesions in CIA mice

Mice in the control group exhibited no redness or swelling in their joints, paws, or toes. In the model group, redness and swelling in the ankle joints were observed in some mice 12 to 15 days after the initial immunization. Following the booster immunization on day 21, most mice developed significant joint swelling, which reached its peak between days 36 and 42. In severe cases, joint deformity and rigidity were noted, Figure 2.

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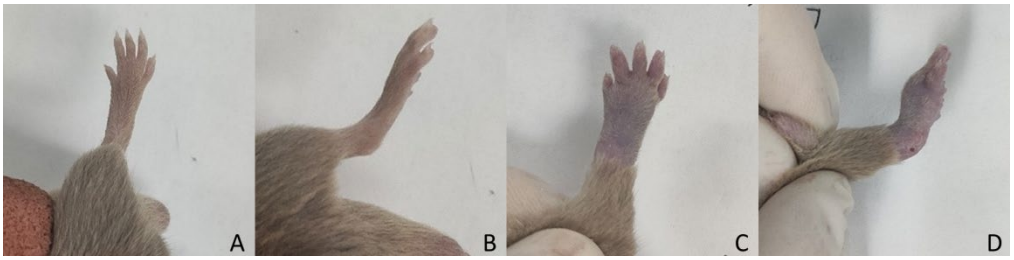

Figure 2: Swelling paws in mice with CIA. Immunization 12d, the paws of some CIA mice appeared red and swelling, the period of swelling peak was from 36 d to 42 d. A, B: normal mice; C, D: CIA mice.

4.3 Scores of arthritis index in CIA mice

Following the initial immunization, arthritis index scores were evaluated every three days. Some mice began to show redness and swelling in their ankle joints between 12 and 15 days. As the arthritis progressed, the arthritis index levels in the mice continued to increase, peaking between 36 and 42 days with an average index value of 10.6. The arthritis index (AI) data revealed that the AI scores of the model group mice were significantly higher than those of the control group ($P < 0.01$), Figure 3.

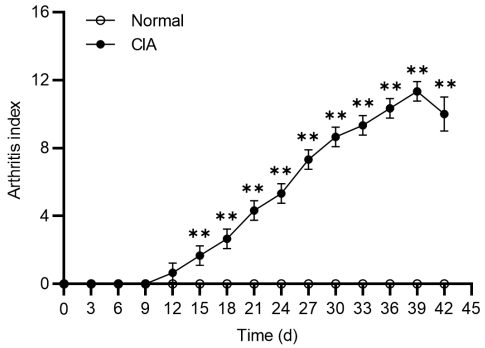

*Figure 3: Scores of arthritis index in mice with CIA. Mean±SD. ** P<0.01 vs normal.*

4.4 Pathological changes of ankle joints in CIA mice

Histological analysis of hematoxylin-eosin (HE) stained sections of ankle joints revealed that the joint surfaces of mice in the control group remained intact, with cartilage appearing light pink and uniformly stained. The joint spaces were maintained at normal levels, and the synovial tissue surface was smooth, showing no signs of abnormal proliferation or aggregation of inflammatory cells. In contrast, the joints of mice in the model group displayed abnormal proliferation and invasive features of the synovial tissue, accompanied by the formation of pannus. Significant infiltration of inflammatory cells was observed within the synovium. Additionally, degenerative changes and necrosis were evident in the articular cartilage, and the underlying bone was damaged, resulting in a narrowed joint space, Figure 4.

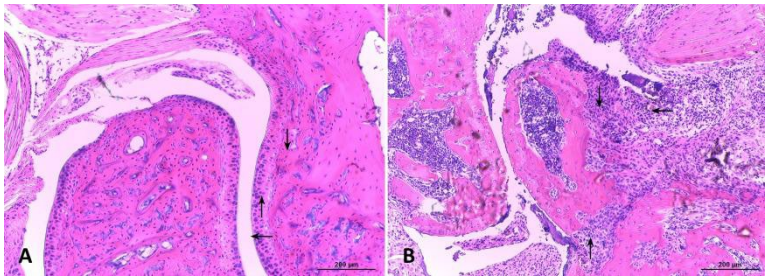

Figure 4: HistologLical pathology of ankle joints in CIA mice (HE staining, ×100). In figure A, there were no inflammatory cells in normal mice, ↑ showed articular cartilage, ↓ showed bone tissue, ← showed ankle space. In figure B, the synovium of CIA mice was hyperplasia, cartilage was destroyed, pannus was formed, and inflammatory cells infiltrated into synovium. ↑ showed cartilage destroyed, ↓ inflammatory cells infiltrated, ← showed pannus.

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4.5 Expression of the PI3K/AKT/mTOR signaling pathway proteins, S100A4 and VEGF in CIA mice

PI3K, AKT, mTOR, S100A4, and VEGF are predominantly localized in the cytoplasm, where they appear as small yellow-brown granules under microscopic observation. Immunohistochemical analysis demonstrated a significant increase in the positive expression of PI3K, AKT, mTOR, S100A4, and VEGF in the synovial tissue of the ankle joints in the model group mice compared to the control group ($P < 0.01$), Figure 5.

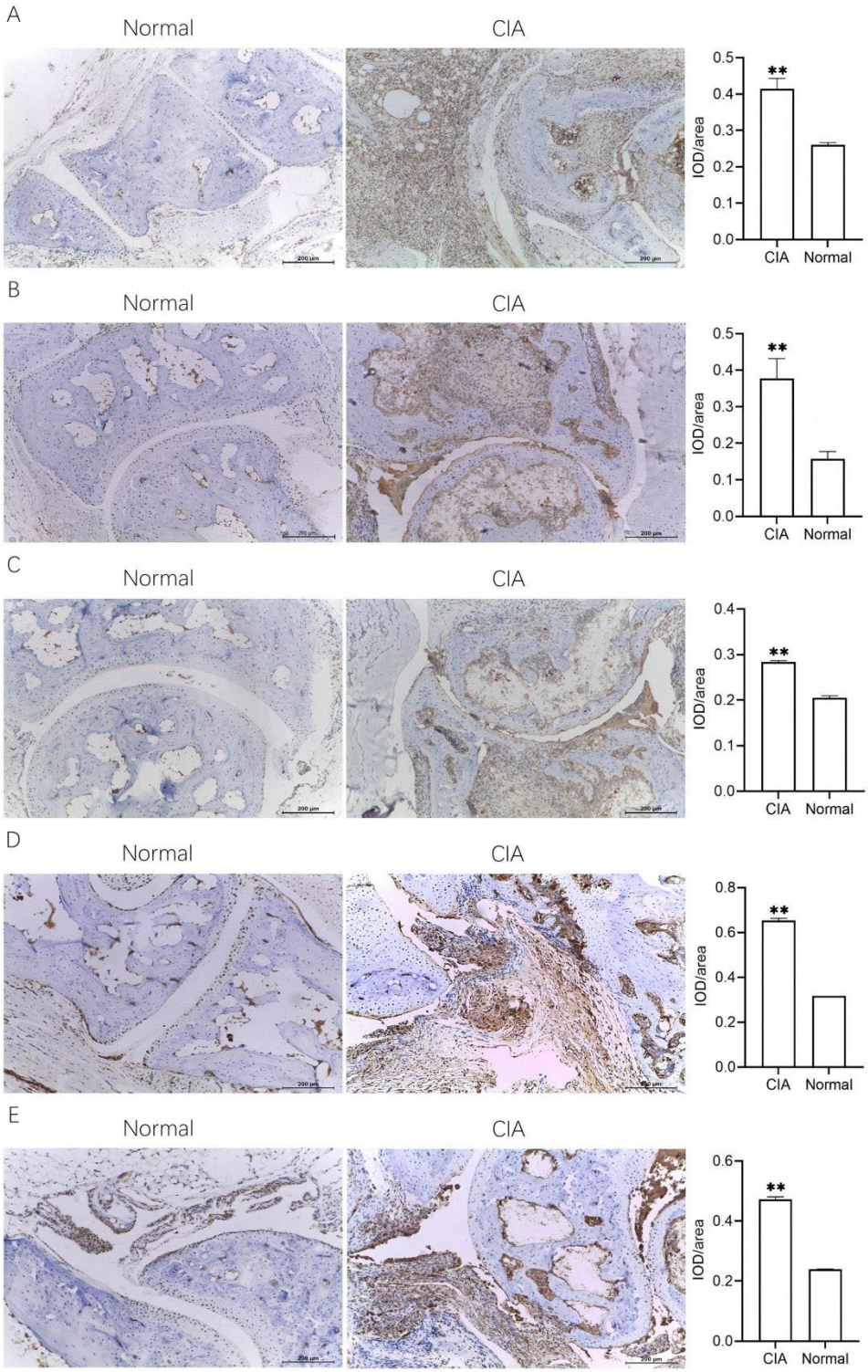

*Figure 5: Expression of PI3K, AKT, mTOR, S100A4, VEGF protein in mice with CIA (immunohistochemical staining, ×100). A: PI3K; B: AKT; C: mTOR; D: S100A4; E: VEGF. Mean±SD. ** P<0.01 vs normal.*

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5. Discuss

The collagen-induced arthritis mouse model serves as an ideal experimental model for studying rheumatoid arthritis. It closely mimics human RA in terms of pathological manifestations, immunological mechanisms, and clinical features[8]. Pathological characteristics include abnormal proliferation and invasive behavior of synovial tissue, accompanied by the formation of pannus. There is significant infiltration of inflammatory cells within the synovium, as well as damage to the articular cartilage and erosive changes in the bone, leading to a narrowed joint space. The CIA mouse model is constructed using a well-established technique with a high success rate, making it widely utilized for studying the pathogenesis of RA and for the development of new drugs[9].

Despite extensive research into the pathogenesis of rheumatoid arthritis, the specific mechanisms of the disease remain incompletely understood. The phosphatidylinositol-3 kinase (PI3K)/protein serine-threonine kinase (Akt)/mammalian target of rapamycin (mTOR) signaling pathway is a well-established signal transduction pathway implicated in the development of rheumatoid arthritis. Research indicates that synoviocytes in RA secrete numerous pro-inflammatory factors, which continuously stimulate these cells, resulting in abnormal proliferation and decreased apoptosis[10]. The overactivation of the PI3K/Akt signaling pathway is recognized as a critical factor contributing to the imbalance of proliferation and apoptosis in fibroblast-like synoviocytes (FLSs). This imbalance, marked by decreased apoptosis, increased proliferation, and abnormal migration, plays a significant role in the progression of RA[11]. Studies have consistently identified elevated expression of PI3K and Akt in the synovial tissue of RA patients[12]. The results of this study show robust positive immunohistochemical staining for PI3K, Akt, and mTOR proteins in the synovial tissue of ankle joints in CIA model mice, indicating elevated expression of the PI3K/Akt/mTOR signaling pathway in this tissue.

S100 calcium-binding protein A4 (S100A4), a low molecular weight protein in the S100 family, has been demonstrated to interact with the RAGE receptor and vascular endothelial growth factor (VEGF). This interaction facilitates cell proliferation and stimulates the formation of new blood vessels[13]. This process can result in inflammatory responses, synovial hyperplasia, pannus formation, and tissue destruction in RA, thereby accelerating disease progression[14]. Our previous research has demonstrated that the expression of S100A4 protein is elevated in the synovial tissue of RA patients. In vitro experiments have shown that S100A4 can stimulate RAFLSs to secrete VEGF. This finding suggests that S100A4 may be a key factor contributing to the recurrent inflammation in the synovial tissue of RA patients[15]. The findings of this study demonstrate that the expression levels of S100A4 and VEGF in the synovial tissue of ankle joints in CIA model mice were significantly elevated compared to the control group. This suggests that the inflammation, synovial hyperplasia, pannus formation, and tissue destruction observed in the synovial tissue of CIA mice may be linked to the increased expression of S100A4 and VEGF.

In summary, the collagen-induced arthritis model, induced by immunizing DBA/1 mice with bovine type II collagen, closely mimics human rheumatoid arthritis in terms of pathological manifestations, immunological mechanisms, and clinical features. This model effectively reflects the pathogenesis of RA and can be assessed using measures such as the arthritis index in mice, changes in body weight, pathological changes in ankle joints, and immunohistochemical analysis.
The elevated expression of PI3K, AKT, mTOR, S100A4, and VEGF in the synovial tissue of CIA mice indicates that the PI3K/AKT/mTOR signaling pathway and S100A4 could be potential therapeutic targets for RA.

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