Collagen-induced Arthritis Osteoporosis model and dynamic changes of osteoimmunology

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Abstract

Objective: This present study aimed to establish collagen-induced arthritis (CIA) osteoporosis mice model and dynamically observe the pathological and immune characteristics of CIA osteoporosis mice.

Methods: Bovine collagen type was chosen to build the CIA model. The corresponding indexes were observed at 6w, 10w, 14w and 18w after successful modelling: Arthritis Index were used to evaluate RA. The structure and pathological changes of bone and joint were observed by pathological section. X ray film was used to observe morphology of bone and joint; And the serum IL-17 and osteoprotegerin (OPG) levels were detected by Elisa. Rt-PCR was used to assess the mRNA levels of IL-17, NFAT, ROR- γ t and Foxp3 in spleen, the mRNA levels of OPG and RANK in bone tissue.

Results: In the model group at 6w after modelling, AI and pedal swelling volume began to be increased gradually. Compared with the normal group, the serum IL-17 level in the model group increased gradually with time, the serum OPG level was considerably decreased, the mRNA levels of IL-17, NFAT, ROR- γ t, *etc.* in spleen tissue increased. However, the level of Foxp3 mRNA in the spleen of model group reduced. The expression of OPG mRNA in bone tissues was dropped. RANK mRNA expression was increased. Bone density of both femur and tibia were decreased gradually with time, leading to osteoporosis. X-ray transmittance of the spine increased with time, and the spine presented water-wavy changes at 14w.

Conclusion: RA mice model successfully induced by bovine collagen type II, and the extension of lesions aggravated over time; Th17 cells results RANKL / OPG and Treg / TH17 imbalance by excessive secreting IL-17, leading to activation of osteoclasts and inhibit the expression of OPG. Therefore, it was involved in the occurrence of osteoporosis in RA in mice.

1. Introduction

Rheumatoid arthritis (RA) is kind of chronic progressive autoimmune disease characterized by synovitis hyperplasia, pannus formation, inflammatory cell infiltration, articular destruction including erosion of cartilage and bone, increasing the risk of disability and fractures¹. The incidence of RA in worldwide is about 0.5–1%, with a female to male ratio of 2:1². In China, the prevalence of RA is estimated to be 0.42%, approximately 500 million patients suffering from RA³. Osteoporosis (OP) is a common clinical complication of RA. It is a kind of systemic multi-system metabolic bone disease characterized by imbalance of bone metabolism, which leads to the change of bone microstructure and the decrease of bone mineral density, resulting in the decreased of bone mass and the increase of bone embrittlement⁴. OP is expected to come up nearly 9 million fractures annually worldwide⁵. The possibility of secondary OP in patients with RA is double the general population. Moreover, OP secondary to RA has a tendency to fracture than primary OP^{6,7}. Osteoporosis will not only lead to a series of related complications, such as fractures, also have activity in patients with serious limitations, which will increase the financial burden of pain and suffering, and reduced quality of life, bring huge challenges to the social economy and medical

resources⁸. Therefore, it is important to put in place an appropriate animal model and find out the mechanism of RA.

Since the background of DBA/1 mice has the highest morbidity of RA, consequently they are the most commonly used strains, the pathological manifestations are proliferative synovitis infiltrated with monocytes and neutrophils, Pannus formation, inflammatory cell infiltration, bone erosion and fibrosis, similar to the clinical-pathological changes of human RA⁹. Type II collagen (Col II) is expressed solely at the joint and articular cartilage. Therefore immunity against heterologous Col II species generated may result in joint destruction and has been explored in numerous studies including mice, rats and monkeys¹⁰. As a consequence, in this study, high purity bovine type II collagen was selected to induce RA to construct an RA osteoporosis model.

In the present study, we induced DBA/1 mice immunized with type II collagen containing CFA to induce RA, due to skeletal and immune systems are inextricably linked. Therefore, we focused on bone immunology, aiming to observe bone immunology, the X-ray of the spine changes and pathological features of RA-OP mice as well as identify the pathogenesis of leading to the OP secondary to RA.

2. Materials And Methods

2.1 Experimental Animals

A total of 100 SPF grade DBA/1(H2q) male mice aged 8w were purchased from Vital River experimental animal Co., LTD, Beijing, China. [production license: SCXK (Beijing) 2012-0001]. The mouse was placed in a specific pathogen free (SPF) room with controlled temperature of $23 \pm 2^{\circ}\text{C}$. All mouse had free access to food and water. This study strictly abided by the welfare ethical principles of animal experimentation.

2.2 Reagents and instruments

Col II was purchased from Shanghai Chondrex Biotechnology Co., Ltd., Shanghai, China; IL-17 and OPG enzyme-linked immunosorbent assay (ELISA) kit was purchased from Beijing R&D systems Co., Ltd., Beijing, China; Fluorescence quantitative PCR instrument was purchased from Bio-Rad, Laboratories, Inc. America; Microplate reader was purchased from Thermo Co., Ltd., America; Model 725 – 86°C low-temperature refrigerator was purchased from FORMA Co., Ltd., America; FX pro multispectral small animal imaging system was purchased from Carestream Co., Ltd., America; Multicolor Real-time PCR Detection System was purchased by Bio-Rad Co., Ltd., America; Tissue Embedder was purchased from MICROM Co., Ltd., Germany; Dyeing machine was purchased from LEICA Co., Ltd., Germany.

2.3 Preparation of CIA model

100 DBA/1 mice were divided into a model group ($n = 68$) and normal group ($n = 32$). Model group using the following method established CIA model: Col II was dissolved in 0.05mol/L acetic acid at $2\text{ mg}\times\text{ml}^{-1}$, 4°C for the night and adding CFA to the collagen, mixed evenly and make $2\text{ mg}\times\text{ml}^{-1}$ collagen emulsion; then 1.2 ml of collagen emulsion was drawn by needle tube, 68 mice were then injected subcutaneously

through the tail. One week later, 0.5 ml collagen emulsion was injected to boost the immunity. 32 mice in normal group did not receive the collagen emulsion in this experiment.

2.4 Dynamic evaluations of the Arthritis Index (AI) and clinical features

In the experiment, AI integral method¹¹ was used for assessment, the mouse model was assessed at 1, 2, 3, and 4 weeks after establishment. Based on the severity of the degree of joint swelling and joint deformity, AI was scored: No pedal swelling and joint deformity, AI was scored: 0 points; Slight swelling of the ankle or toe: 1 point; Severe redness of joints or toes: 2 points; Joint stiffness or rigidity: 3 points. Each mice's paws were observed separately to record their cumulative scores. There were a highest of 12 points for four paws of each mice. At week 4 of modelling, 32 model mice with an arthritis score of 6 or higher were selected, and then randomly divided into four groups: the 6w group; the 10w group; the 14w group and the 18w group, with 8 mice in each group, the normal group was also divided into 6w group; 10w group; 14w group and 18w group. As well as body weight and clinical traits were observed respectively at week 6, 10, 14 and 18.

2.5 Radiological Assessments

The ankle joint and pedal of the mouse in RA model group and normal group were measured at 6w, 10w, 14w and 18w after modelling, Small animal X-ray machine was used to take pictures of ankle and foot joints of mice for imaging examination and analysis.

2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

Serum levels of IL-17 and OPG were detected according to the manufacturer's introduction at 6w, 10w, 14w and 18w after modelling. Normal group and model group were measured respectively.

2.7 Real-Time PCR

Trizol reagent was used to extract total RNA from bone tissue, and the cDNA was reversely transcribed according to the instructions using the Prime Script RT reagent kit. Real-time PCR analysis was performed with the BioRad CFX96 Touch TM System using iQTM SYBR1 Green Supermix. Relative gene expression was computed by the $^{-\Delta\Delta C_t}$ method. The sequence of primers (forward and reverse) was as follows: Foxp3, forward CCCATCCCCAGGA GGAGTCTTG, reverse ACCATGACTAGGGGCACTGTA; IL-17, forward TTAACTCCCTT GGCGCAAAA, reverse CTTTCCCTCCGCATTGACAC; ROR- γ t, forward GACCCACACCTC ACAAATTGA, reverse AGTAGGCCACATTACACTGCT; NFAT, forward GGAGCGGAGAA ACTTTGCG, reverse GTGACACTAGGGGACACATAACT; OPG, forward ACCCAGAAACT GGTCATCAGC, reverse CTGCAATACACACTCATCACT; RANK, forward GGACGGTG TTGCAGCAGAT, reverse GCAGTCTGAGTTCCAGTGGTA; β -actin, forward CATCCGTAA AGACCTCTATGCCAAC, reverse ATGGAGCCACCGATCCACA.

2.8 Histological Analysis

The femoral tissue segment was fixed in 10% neutral formaldehyde buffer, decalcified in 10% EDTA bone decalcifier, gradient ethanol dehydration, transparency of xylene and embedded in paraffin. The five-micrometre tissues section was prepared and stained with hematoxylin-eosin (HE). The structural changes of cartilage and trabecular bone of the femur were observed under an optical microscope.

2.9 Statistical Analysis.

All data are analyzed with Prism software (GraphPad Software, Inc.) and expressed as means $\pm$ SEM. The comparison of quantitative data between the two groups was performed by T-test, $P < 0.05$ was considered statistically significant difference.

3 Results

3.1 Histopathological damage in CIA mouse

HE staining at the knee joints indicated healthy knee joint without synovial inflammation, synovial cells proliferation, pannus formation and osteoporosis in all normal group mouse (Fig. 1A and 1B). In the 6w group, there are large number of inflammatory cell infiltration in the knee joints, with cartilage thickened, accompanied by bone and cartilage erosion (Fig. 1C and 1D). In the 10w group, the knee joints of mice showed inflammatory cell infiltration, invasive pannus formation, cartilage and bone erosion, synovial hyperplasia, cartilage and bone erosion, joint destruction. In the 14w group, there was large inflammatory cell infiltration in the joint cavity, and a lot of fibrosis in the bone marrow, synovial hyperplasia around the joint is abnormally hypertrophy, fibrous tissue hyperplasia, cartilage and soft tissue damage, the subchondral bone is also filled with inflammatory cells, the normal structure is severely damaged (Fig. 1G and 1H). In the 18w group, the normal structure of the knee joint was completely lost, with severe bone erosion, blurred cartilage surface, absorbed trabecular bone, and there was fibrous necrotic material filling in joint cavity, joint space disappears, joint fusion occurs, surrounding soft tissue thickens, new bone formation in periosteum leads to joint rigidity and deformity (Fig. 1I and 1J).

3.2 CIA mouse manifestations and RA scored

After immunization of DBA/1 mice with Col II in CFA, anterior pedal and posterior pedal were swollen and redness in CIA model mice, which didn't like to move, limited movement and weight loss. The body weight of CIA model mice at 6w, 10w, 14w and 18w were dramatically lower than that in the same period normal group ($P < 0.01$) (Fig. 2A). At week 6, successive hyperemia on the joint surface was observed in the CIA model group, which the score of rheumatoid arthritis was significantly upward trend than that in the normal group and score peaked at 18w ($P < 0.01$) (Fig. 2B). The swelling degree of the left paw and left hind paw of CIA mice raised with the increase of modelling time ($P < 0.01$) (Fig. 2C-D).

3.3 Serum levels of IL-17 and OPG, mRNA relative expression of IL-17, NFAT, FoxP3, ROR- γ t, OPG and RANK.

Compared with the same period normal group, the serum IL-17 level of mice in the CIA model group were increased considerably at 14w and 18w ($P < 0.01$), and it added with the increase of modeling time after 10 weeks ($P < 0.01$) (Fig. 3A). IL-17 mRNA expression in the spleen was considerably increased in the CIA model group at 14w and 18w ($P < 0.05$) (Fig. 3D). Compared with the normal group, the serum OPG level were significantly decreased in the CIA model group at 6w, 10w, 14w and 18w ($P < 0.01$) and CIA model group began to decline gradually from week 10, 10w and 14w, 14w and 18w in CIA model group were statistically significant, respectively ($P < 0.01$) (Fig. 3B). Compared with the normal group, the relative expression of Foxp3 mRNA were decreased in the spleen in the model group, which was statistically significant at week-6 and week-14 ($P < 0.01$) (Fig. 3C). ROR- γ t, master regulators of Th17 cell, mRNA expression in spleen slowly were increased over time, compared with the normal group in the same period, the 14w CIA model group and the 18w CIA model group being increased significantly, ($P < 0.05$) (Fig. 3E). The mRNA expression of NFAT in spleen also increased with time, compared with the normal group of the same period, being statistical significance at week-18 ($P < 0.01$) (Fig. 3F). Compared to the normal group, the relative expression of OPG mRNA in the bone of the mouse in the model group were decreased over time and significantly reduced at 14w and 18w ($P < 0.05$) (Fig. 3G). On the contrary, the relative expression of RANK mRNA in the bone tissue of the CIA model group increased with time, at 18w was statistically significant ($P < 0.05$) (Fig. 3H).

3.4 The radiological signs of spinal destruction, mineral density and osteoporosis in CIA mouse

The radiographic results from the CIA model group mouse manifested that spinal deformities increase with time and change in water ripples at week-14 and week-18, the transmittance of X-ray film increased with time and osteoporosis were increased at 6w, 10w, 14w and 18w (Fig. 4B-F), in contrast with the normal group (Fig. 4A). Bone density of both femur and tibia were decreased gradually with time, the bone mineral density of femur has been reduced significantly more than that of the tibia in the CIA model group at 10w and 14w. Compared with the normal group, femur mineral density of CIA model group at 6w, 10w, 14w and 18w were significantly reduced ($P < 0.01$) (Fig. 4G). Tibial mineral density of CIA model group at 14w and 18w was reduced considerably, ($P < 0.01$) (Fig. 4H).

4. Discussion

The inflammatory response of RA and the activation of the immune system leading to abnormal bone tissue metabolism, which can lead to increasing bone resorption, reduced bone formation, decreased bone density, and osteoporosis and fractures^{12,13}. This disorder is most typical among women and the elderly, uncontrolled active RA leads to osteoporosis, joint destruction, impaired mobility and cardiovascular disease and other complications¹⁴. In the experiments, we immunized DBA / 1 mice with Col II collagen in CFA to establish a CIA mouse model, which has both immunological and pathological features of RA. The pathological changes in Fig. 1 are inflammatory cell infiltration, proliferative synovitis, bone erosion with destruction of articular cartilage, and the condition worsens with time, prompting that our RA model was successfully established. Severe OP leads to compression fractures and deformities in

the spine, as well as nerve compression, affecting lower limb activities¹⁵. As shown in Fig. 4, An x-ray of the CIA mouse revealed deformities in the spinal and X-ray transmittance increased with time. Bone density decreased over time, leading to osteoporosis and increased risk of fragility fracture. Generalized bone loss is the leading cause of fracture in RA patients. This loss of bone density occurs quietly and continuously. Studies have shown that the destruction of RA articular cartilage and bone, and secondary osteoporosis is nearly related to the formation and activation of osteoclasts¹⁶⁻¹⁸. Osteoblast (OB) and osteoclast (OC) are the two main types of cells that maintain dynamic balance of bone. Under normal circumstances, OB and OCs are in a dynamic equilibrium state *in vivo*. Therefore, bone formation and resorption are in a balanced state, which is determined by the balance of OPG and RANKL^{19,20}. RANKL-RANK-OPG is a molecular bridge between bone and the immune system, RANKL receptor, nuclear factor kappa-B receptor activator (RANK) is expressed in monocyte-macrophage osteoclast precursor cells and mature osteoclasts, after RANKL binds to RANK, it induces OC precursor cells to differentiate into OC, and also promotes OC's bone resorption activity, inhibits OC apoptosis and prolongs OC life^{21,22}. Osteoprotegerin (OPG), a soluble decoy receptor of RANKL, can specifically bind to RANKL, thereby competitively inhibiting RANKL-RANK binding and inhibiting RANKL activity²³. OPG is also a member of the tumor necrosis factor receptor family, which can block tumor necrosis factor, induce osteoblast apoptosis *in vivo*, and reduce the bone loss of tumor necrosis factor-transgenic mice²⁴. In the experiments, from the results in Fig. 3, the RANK of mice in the CIA model group increased with time, and OPG decreased with time. The process of homeostasis is broken. Osteoclast activity prevails, which can result in RA.

The immune system, including CD4⁺ T cells and inflammatory cytokines, is a crucial regulator of bone formation and bone resorption balance. Th17 cells are CD4⁺ T cells, secreted explicitly under the control of the nuclear transcription factor ROR- γ t of IL-17. IL-17 is a cytokine promoting and inducing of T lymphocytes, and promoting immune response and inflammation development²⁵. Tyagi *et al*²⁶ demonstrated that IL-17 can directly stimulate OC differentiation, inhibit bone matrix mineralization, inhibit OB differentiation, increase the secretion of RANKL by OB. Moreover, in the collaborative culture of bone marrow stromal cells with RANKL and M-CSF, the differentiation of bone marrow stromal cells towards OC could be induced. In the experiments, from the results in Fig. 2, IL-17 serum level, and spleen expression of mice in the CIA model group increased with time, and the condition became more serious. Treg cell is a subgroup of CD4⁺ T cells with immunosuppressive activity, inhibiting T cell over-activation and OC formation, inducing immune incompetence, and maintaining autoimmune tolerance, which inhibits the inflammatory response to relieve RA symptoms^{27,28}. Transcription regulator Foxp3 is regarded as specific lineage-specific marker for Treg cells and its sustained expression is a critical factor in keeping Treg repressing activity²⁹. More studies have found that the change of the transcription factor ROR γ t / Foxp3 balance in T cells fundamentally determines the shift of Th17 / Treg differentiation, and then affects the occurrence and progress of RA³⁰. As shown in Fig. 3, nuclear transcription factor ROR- γ t mRNA expression of spleen in CIA model group increased over time, IL-17 secreted by TH17 cells also increased, Treg transcription factor Foxp3 mRNA expression of spleen in CIA model group decreased over

time. The reason may be the decrease of CD4⁺ Treg, which makes the negative immune regulation mechanism disorder in RA patients, and cannot inhibit the excessive activation of T cells, coupled with the powerful inflammatory cells cytokine IL-7, also able to up-regulate the expression of RANKL in OC, and increases the Osteoclast Precursor cells (OCPs) expression of RANK, increasing the sensitivity of OCPs to RANKL, potentiates the osteoclastogenic activity, thereby disrupting the balance of RANKL/OPG in synovial fluid and aggravating bone destruction and resorption^{31–33}. Resulting in RA and secondary OP.

5. Conclusion

The dynamic balance of bone tissue is governed by the immune system, bone destruction due to activation of the immune system. The immune system of RA mice releases IL-17 cytokines excessively, affecting the balance of RANKL / OPG and Treg / TH17, leading to an imbalance of bone immunity, inhibits the formation of osteoclasts and promotes osteoclast activation, thereby result in bone erosion and osteoporosis. However, the relevant regulatory mechanisms are still required to be determined by further experiments. This study serves as an experimental basis for in-depth study of rheumatoid arthritis, and offers new inspiration and models for the treatment of rheumatoid arthritis osteoporosis complications.

Declarations

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Authors' contributions

YGD, XL, ZQS, and YYL carried out the animal experiments. LJ and YQC participated in the Immunological index indicator, LJ and ZQS completed Radiographic assessment. QYS, HYF and DWX involved in the design of this research and took part in its coordination. WM performed the statistical analysis and prepared for the manuscript. All authors have read and approved the manuscript.

Statement

We confirmed that all methods were performed in accordance with the relevant regulations of the Animal Committee of the Zhjiang province Admininstration Rule of Laboratory Animal (reference number: ZSL-2015-52), and the study was carried out in compliance with the ARRIVE guideline.

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Availability of data and materials

The original contributions presented in the study are in the article Material, further inquiries can be directed to the corresponding authors.

Ethics approval and consent to participate

The in vivo protocols were approved by the Animal Committee of the Zhjiang province Admininstration Rule of Laboratory Animal.

Consent for publication

Not applicable.

Conflict of interest statement

The authors declare no competing financial interests concerning this article.

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Figures

Figure 1

Representative histopathology of the knee joint in CIA mice. (A). The normal knee joint structure of DBA/1 mice (100×) (B). the magnification at the dotted line of A (400×); (C), (E), (G), (I) was the knee joint pathology section of CIA mice at 6, 10, 14, and 18w respectively (100×). The boxed area in Figure (C), (E), (G), (I) denotes the region shown in Figure (D), (F), (H), (J) (400×). Legend: A, angiogenesis; B, bone; BE, bone erosion; C, cartilage; CE, cartilage erosion; P(400×), invasive pannus formation; S, synovial layer; and

SH, synovial hyperplasia, Articular cartilage, both mineralized and nonmineralized, is lost in large segments (black dashed line). A–J were stained with HE.

Figure 2

CIA mouse manifestations and CIA scored. CIA was induced by bovine type I collagen and complete Freund's adjuvant. (A) The body weight of each group mouse. (B) RA scored was evaluated as describing. Data shown are means $\pm$ SD of 8 mice in each group. * P <0.05, ** P <0.01 versus the same period normal group. (C-D) Comparisons of the left paw and hind paw swelling volume at 0w, 6w, 14w and 18w after modelling. Data shown are means $\pm$ SD of 8 mice in each group. * P <0.05, ** P <0.01 versus the 0w group.

Figure 3

Serum level of IL-17 and OPG, RT-PCR analysis of mRNA expression of IL-17, NFAT, FoxP3, ROR-γt, OPG and RANK. (A) Levels of IL-17 in the serum. (B) Levels of OPG in the serum. Data presented as mean ± SEM of 8 mice in each group. * $P < 0.05$, ** $P < 0.01$ versus same period normal group, # $P < 0.05$, ## $P < 0.01$ versus CIA model group. (C-H) Relative mRNA expression of IL-17, NFAT, FoxP3, ROR-γt in spleen and OPG, RANK in the bone. β-actin was used as internal control. Data presented as mean ± SEM of 4 mice in each group. * $P < 0.05$, ** $P < 0.01$ versus same period normal group.

Figure 4

Conventional radiograph and bone density in each group. A-E Representative radiograph of the normal mice; 6w CIA mice; 10w CIA mice; 14w CIA mice; 18w CIA mice. G-H Bone density of femur and tibia in each group. Data presented as mean $\pm$ SD of 8 mice in each group. * $P < 0.05$, ** $P < 0.01$ versus same period normal group.