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Original Article

Hylonome

Early closure of growth plate causes poor growth of long bones in collagen-induced arthritis rats

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Abstract

Abnormalities of the epiphyseal growth plate that occur in collagen-induced arthritis (CIA) were studied. CIA was induced in 6-week-old Lewis rats by immunization with type II collagen. Radiographic examination revealed the early closure of the epiphyseal growth plate with growth retardation of the femur and tibia. Histological evaluation confirmed the early closure of the epiphyseal growth plate accompanied by decreased intensity of safranin-O staining indicating decreased amounts of proteoglycans in the extracellular matrix (ECM) of the cartilage. Immunohistochemical methods showed that the number of chondrocytes expressing matrix metalloproteinase (MMP)-3 and/or vascular endothelial growth factor (VEGF) increased in the growth plates of CIA rats. This study confirmed that disturbances of long bone growth with early closure of the epiphyseal growth plates occur in CIA. There appeared to be overexpression of MMP-3, which may be involved with proteoglycan degradation. Additionally, VEGF, which is associated with cartilage ossification and angiogenesis, might also play a role in this event. Further clarification of the mechanism of the growth disturbance in CIA may yield clinical benefits, especially in prevention of the premature closure of growth plate that is seen in juvenile rheumatoid arthritis and other diseases.

Keywords: Epiphyseal Growth Plate, Collagen-Induced Arthritis, Rat, Matrix Metalloproteinase, Vascular Endothelial Growth Factor

Introduction

Experimental animal models, such as collagen-induced arthritis (CIA) and adjuvant arthritis, are widely used to examine pathologies of arthritis with immunological abnormalities and evaluate efficacy of therapeutic agents[1-3]. However, few studies have focused on pathological changes in the epiphyseal growth plate in animal models of inflammatory chronic arthritis. Jee et al.[4] reported a decreased thickness and erosion of the epiphyseal growth plate following adjuvant-induced polyarthritis in rats. This was accompanied by an infiltration by inflammatory cells and marked proliferation of osteoclasts. Bunger et al.[5] reported that in carrageenan-induced monoarthritis there were growth disturbances in the bones adjacent to the involved joints. Although these reports indicate that growth abnormality is one of the changes that may develop in rat models of arthritis, details of pathological changes that occur in the growth plates and associated pathogenesis remain unclear.

Juvenile rheumatoid arthritis, juvenile chronic arthritis, and hemophilic arthropathy are sometimes associated with premature closure of juxta-articular epiphyses, resulting in joint malalignment, extremity length discrepancy or short stature[6]. Although inflammatory changes may contribute to the pathogenesis of early epiphyseal closure in such clinical cases, the causality of this relationship has not been proven.

The purpose of this study was to determine if growth retardation occurs at the epiphyseal plates in CIA rats, and to describe the histopathology of the growth plate in this model.

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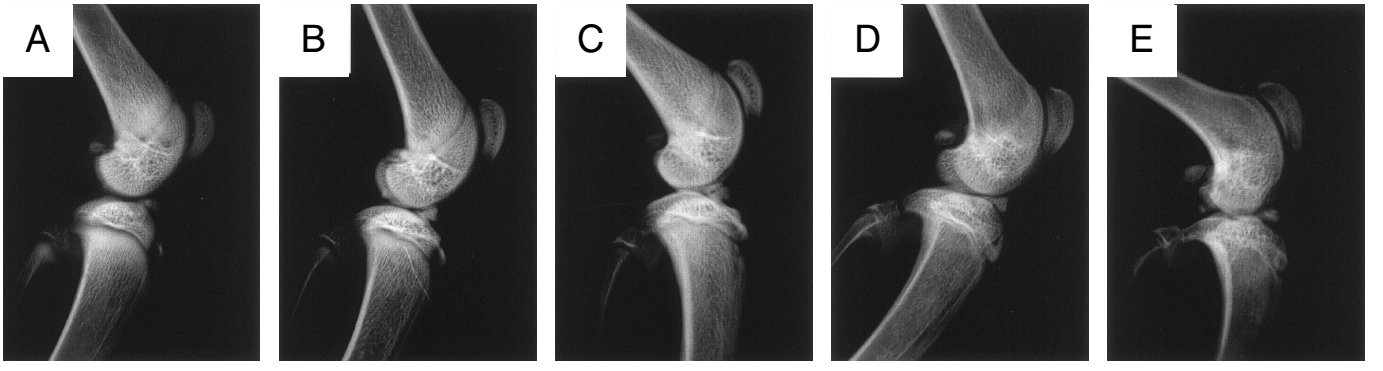


6-week-old normal rat | 12-week-old normal rat | 18-week-old normal rat | 12-week-old CIA rat 6 weeks after immunization | 18-week-old CIA rat 12 weeks after immunization

Figure 1. Radiologic appearance of the knees. Growth plates gradually grew narrower with age, but were still visible in 18-week-old normal rats (A-C). In CIA rats, growth plates rapidly narrowed, and had closed (radiographically observed) 12 weeks after immunization (D, E).

Materials and methods

Induction of CIA

CIA was induced using the modified method described previously[7]. Six-week-old female Lewis rats (Clea Japan, Tokyo, Japan) were immunized intradermally with 0.5 mg of bovine type II collagen (Cosmo Bio, Tokyo, Japan) emulsified in 0.5 ml of Freund's incomplete adjuvant (Difco, Detroit, MI, USA) at 4°C. Seven days after the first immunization, the rats received an intradermal booster injection of half the initial volume of emulsified type II collagen. CIA was detected in all of the animals by day 12-16 after the first immunization. Twenty CIA and 20 normal (control) rats were used in this study.

Radiographic evaluation of the knee and measurement of femoral and tibial length

Twelve CIA and 12 normal rats were used for radiographic and histological evaluations. Two rats from each group were killed by an overdose of anesthesia sequentially at 0, 2, 3, 4, 6 and 12 weeks. The legs were excised, and bones were separated from soft tissues and placed on a radiographic box. Radiography was performed with μFX1000 (Fuji Photo Film, Tokyo, Japan), with 35kV exposure for 12 seconds (Figure 1A-E).

Femoral and tibial lengths of the remaining 8 CIA and 8 normal rats were measured, as previously described[8], under anesthesia every week until 12 weeks after immunization using a FUJIX BIO-IMAGING ANALYSER system (Fuji Photo Film, Tokyo, Japan). The X-ray image was acquired using an Image Reader and displayed on a computer, and lengths of each femur and tibia were measured with an Image Gauge (Figure 2A).

Tissue preparation

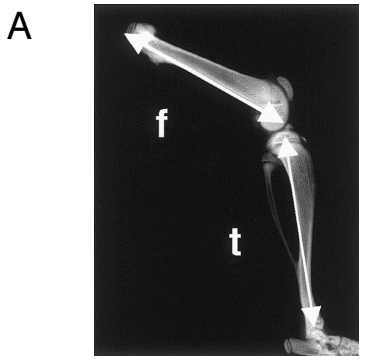
Preparation of tissue samples was performed as follows. After radiographic evaluations, excised legs were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). The femurs and tibias were decalcified in 20% EDTA with microwave irradiation for 5 hours, dehydrated in ethanol and embedded in paraffin. Sagittal sections of knee were cut with the plane of section oriented parallel to the longitudinal axis of the bone. Sections 5μm thick were cut with a microtome and stained with hematoxylin and eosin (H & E), (Figure 3). Serial sections were prepared for safranin-O staining (Figure 4).

Immunohistochemistry for MMP-3 and VEGF

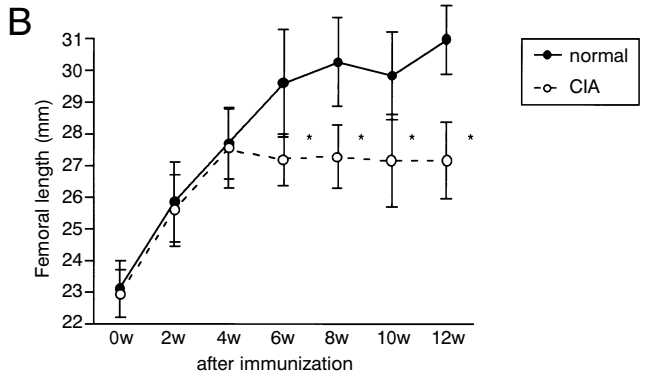
Immunohistochemistry was performed using the streptavidin-peroxidase method with Histofine SAB-PO kits (Nichirei, Tokyo, Japan) according to the manufacturer's protocol. Tissue sections were deparaffinized and hydrated in PBS (pH 7.4), then incubated in 0.3% H_2O_2 in methanol for 30 minutes at room temperature to block endogenous peroxidase activity. After a PBS wash, the sections were incubated for 30 minutes at room temperature with 10% normal serum from the same species as the secondary antibody to minimize background staining, then with primary monoclonal antibodies (anti-MMP-3; Fuji Chemical, Toyama, Japan and anti-VEGF; Upstate Biotechnology, Lake Placid, NY, USA) overnight at 4°C. After washing in PBS, the sections were incubated with secondary antibody for 30 minutes at room temperature, then washed in PBS. The color reaction was performed using the substrate reagent 3,3'-diaminobenzidine tetrahydrochloride (Dojindo, Tokyo, Japan). Finally, the slides were counterstained with hematoxylin, dehydrated in graded ethanol series and mounted.

Morphometric analysis of the growth plate

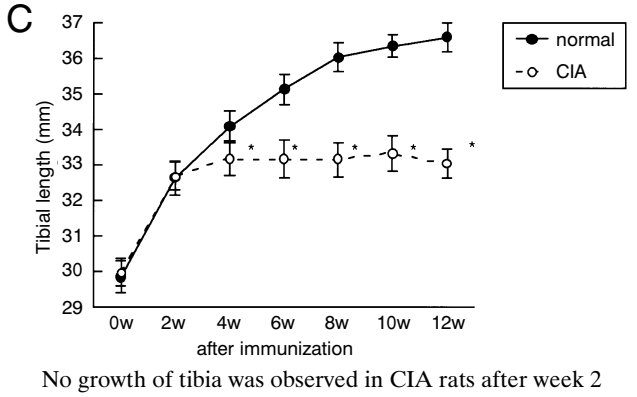
Morphometric analysis was performed as described previously[9-11]. The H & E-stained sections of 4 legs from each group were examined by light microscopy (Nikon ECLIPSE



Femoral length (arrow f) and tibial length (arrow t) were measured radiographically.



No growth of femur was observed in CIA rats after week 4 (solid line, normal; dotted line, CIA).



No growth of tibia was observed in CIA rats after week 2 (solid line, normal; dotted line, CIA).
(Values shown are mean ±SD. *=p<0.01, versus normal rats.)

Figure 2. Length of femur and tibia at times following immunization.

E1000; Nikon, Tokyo, Japan) at 200x magnification, and the microscopic images were displayed on a computer. The widths of the whole growth plate, proliferating zone, and hypertrophic zone of the proximal tibial growth plate were measured at 3 sites for each section using Photoshop (Adobe Systems Incorporated, CA, USA). The mean widths were then calculated (Figure 7A).

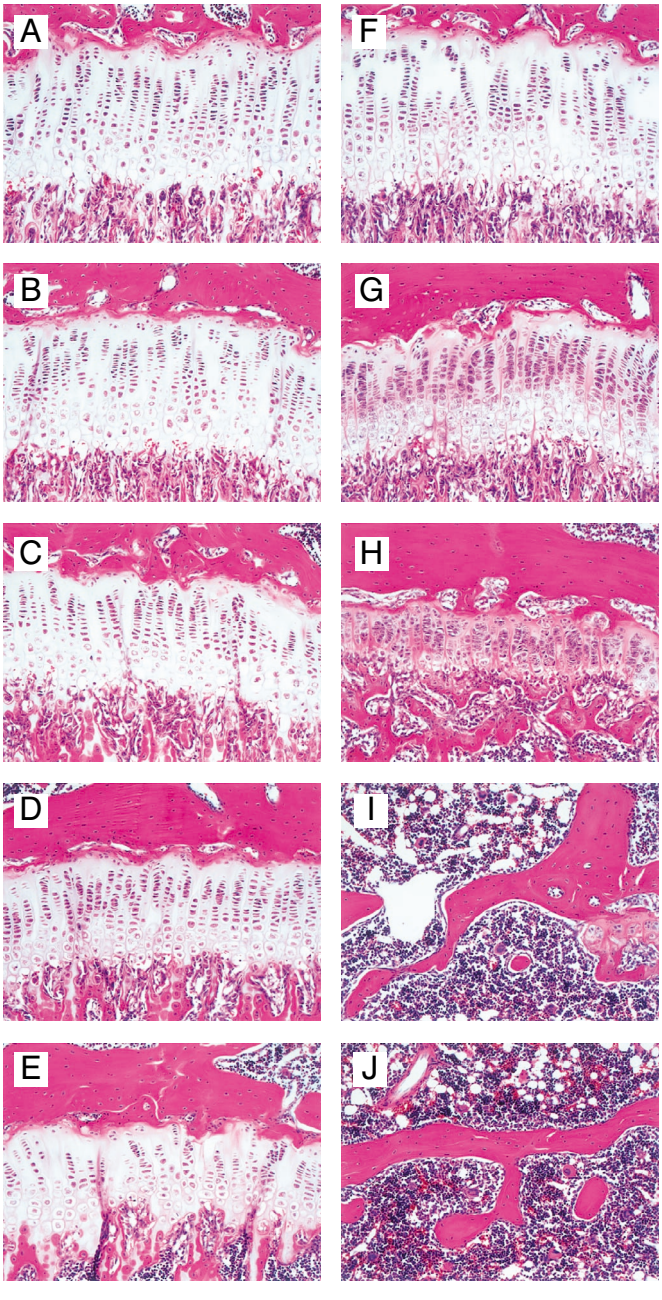


Figure 3. Growth plate proximal tibia (hematoxylin and eosin, 200x).
(A-E) Normal rats. The epiphyseal growth plate of the proximal tibia was composed of very regular columns in the 8-week-old normal rats. The width of growth plate decreased slowly, but the regular arrangement and properties of cells in the column were well conserved until 18 weeks of age (A, 8 weeks old; B, 9 weeks old; C, 10 weeks old; D, 12 weeks old; E, 18 weeks old).
(F-J) CIA rats. Nearly normal column formation in growth plate was observed in the CIA rats at 2 weeks after immunization (age, 8 weeks) (F). The proliferating zone cells appeared swollen at 3 weeks after immunization (G). The width of the hypertrophic cell zone was greatly reduced at 4 weeks after immunization (H). The width of the hypertrophic cell zone was then reduced until it finally disappeared at 12 weeks after immunization (age, 18 weeks) (J). (F, 8 weeks old; G, 9 weeks old; H, 10 weeks old; I, 12 weeks old; J, 18 weeks old).

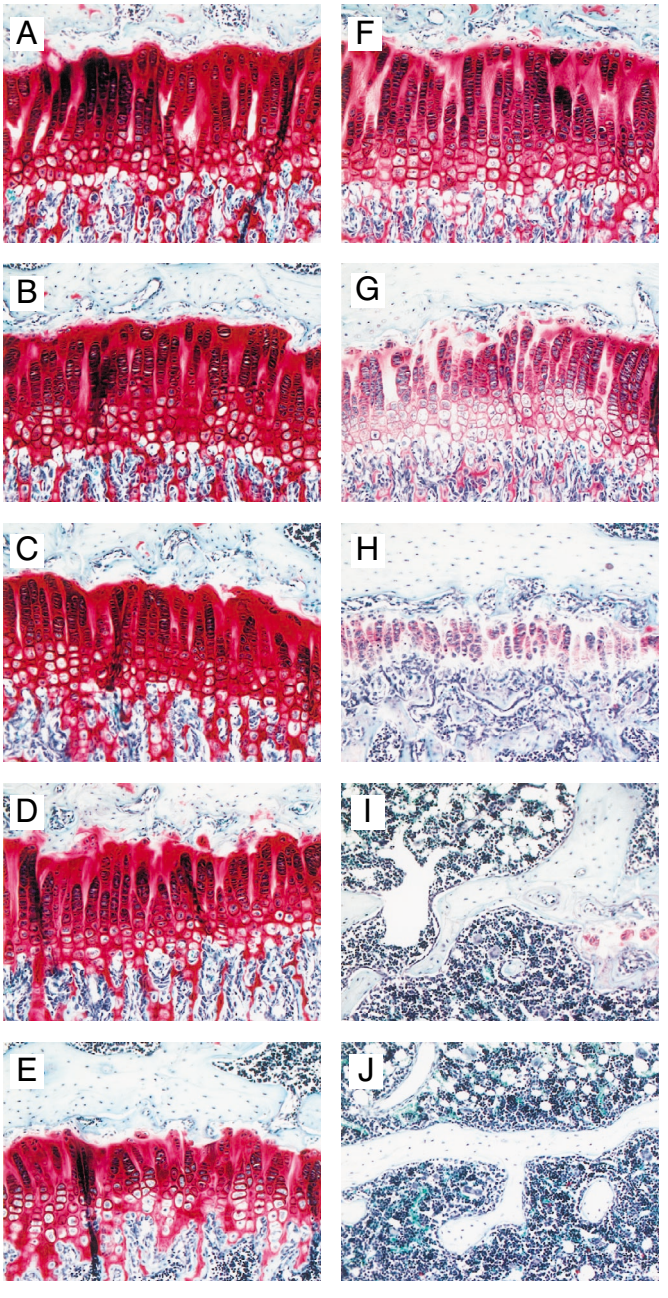


Figure 4. Growth plate of proximal tibia (safranin-O staining, 200x). (A-E) Normal rats. (A, 8 weeks old; B, 9 weeks old; C, 10 weeks old; D, 12 weeks old; E, 18 weeks old).
(F-J) CIA rats. (F, 8 weeks old; G, 9 weeks old; H, 10 weeks old; I, 12 weeks old; J, 18 weeks old).
Intensity of safranin-O staining was well conserved from 8 to 18 weeks of age in the normal rats (A-E), whereas it was decreased at 8 and 9 weeks of age in CIA rats (2 and 3 weeks after immunization, respectively).

Statistical analysis

All data were expressed as the mean ± SD, and statistical analysis was performed with the Mann-Whitney U test. Differences were considered significant if p value was less than 0.01.

Results

Epiphyseal growth plate closure in CIA rats

In normal (control) rats, the growth plate widths gradually decreased with age, from 6 to 18 weeks (Figure 1A-C). The growth plate widths decreased more rapidly with age in the CIA rats and were essentially closed by 18 weeks (Figure 1D and E).

Length of femur and tibia

Femoral lengths of the normal and CIA groups increased in a similar manner until 4 weeks after immunization. After six weeks substantial differences were observed between the normal and CIA groups. In the normal group, femoral length continued to increase throughout the experiment, but the femoral length of the CIA group was nearly constant from 4 to 12 weeks after immunization (Figure 2B).

In CIA rats, the growth curve of the tibia was similar to that of the femur. In normal rats, tibial length increased throughout the experiment, while in the CIA rats there was little increase from 2 to 12 weeks after immunization (Figure 2C). There were significant differences in femoral length after 6 weeks and tibial length after 4 weeks between normal rats and CIA rats (Figure 2B and C).

Histology of the growth plate

The epiphyseal growth plate of the proximal tibia was composed of very regular columns[12]. In normal rats, the width of the growth plate decreased with age, but the regular arrangement and properties of cells in the columns remained well conserved through the experimental period (18 weeks of age) (Figure 3A-E).

In the CIA rats, the growth plates appeared essentially normal for the first 2 weeks after immunization (age, 8 weeks) (Figure 3F). Beginning at 3 weeks, there were striking alterations in the morphology of the epiphyseal growth plate of the CIA rats (Figure 3G). The proliferating cells appeared enlarged, and columns of chondrocytes had become irregular. The width of the hypertrophic zone was significantly less in CIA rats compared with the normal rats. In the CIA rats, the hypertrophic zone was no longer evident by 4 weeks after immunization (Figure 3H), and the epiphyseal growth plate was almost gone by 6 weeks after immunization and was essentially absent by 12 weeks after immunization (Figure 3 I and J).

There were also differences in safranin-O staining of the growth plate between normal and CIA rats. In normal rats, intensity of safranin-O staining remained at the same level from 6 to 18 weeks of age (Figure 4A-E), whereas, in CIA rats, the intensity decreased beginning 2 weeks after immunization (age, 8 weeks) (Figure 4F-J).

Immunohistochemical evaluation of growth plate

Immunohistochemical detection and localization of MMP-3 and VEGF showed that these proteins were expressed in hypertrophic chondrocytes in the growth plates from 8-week-old normal and CIA rats at 2 weeks after immunization (Figure 5A, 5C, 6A and 6C). By 3 weeks after immunization (age, 9 weeks), significant differences were seen between normal and CIA rats. In normal rats, these proteins were detected only in hypertrophic chondrocytes (Figure 5B and 6B), while the enlarged chondrocytes in the proliferating zone from CIA rats also expressed these proteins. The relative number of chondrocytes expressing these proteins increased in the CIA rats (Figure 5D and 6D).

Widths of whole growth plate and proliferating and hypertrophic zones

In the normal rats, the widths of the whole growth plate and hypertrophic zone decreased with age, but growth plate was still evident at 18 weeks of age (same age as CIA rats at 12 weeks after immunization). In the CIA rats, there were rapid decreases in growth plate width with age, especially in the hypertrophic zone. The widths of the proliferating zone also decreased with age, but less rapidly than the hypertrophic zone. At 6 weeks after immunization (age, 12 weeks), growth plate had essentially disappeared (Figures 7B-D).

Discussion

Although CIA is a widely used experimental model of rheumatoid arthritis[13-16], few studies have focused on the pathological changes in the epiphyseal growth plate[4]. In this study, we demonstrated that growth disturbances of long bones occurred during the development of arthritis in the CIA rat model. The longitudinal growth retardation was recognised radiographically in the tibia and femur from 4 weeks after immunization, and the pathological epiphyseal closure was recognised radiographically and histologically at 6 weeks after immunization. Finally, at 12 weeks after immunization, tibial and femoral average lengths in the CIA rats were 3.8mm (12.2% of normal length) and 3.6 mm (9.7% of normal length), respectively, shorter than those in the control rats. These results indicated that pathological epiphyseal closure occurs in CIA rats, and resulted in longitudinal growth retardation of the tibia and femur.

A decrease in safranin-O staining of the growth plates in the CIA rats was evident prior to morphological changes in the chondrocytes. There was a later diminution of the growth plate width, particularly evident in the hypertrophic zone. Since the intensity of safranin-O staining correlates with the amounts of proteoglycans in cartilage[17], the reduced staining of the growth plates in the CIA rats suggests a decreased production and/or an increased degradation of proteoglycans. MMP-3 is a stromelysin, which degrades proteoglycans[18,19]. In this study we showed an increase of MMP-3 producing cells in the growth plates of CIA rats. These findings suggest that over expressed MMP-3 could result in increased proteoglycan degradation in the growth plates of CIA rats.

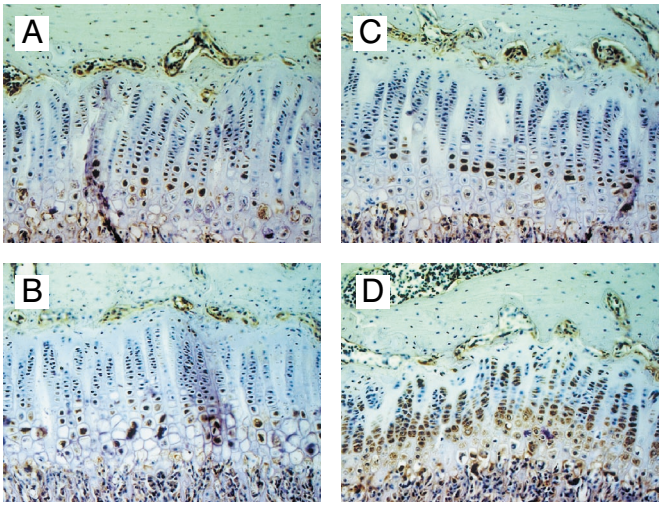


Figure 5. Growth plate of proximal tibia (immunohistochemistry against MMP-3, 200x).
(A, B) Normal rats. (A, 8 weeks old; B, 9 weeks old).
(C, D) CIA rats. (C, 8 weeks old; D, 9 weeks old).
Immunohistochemistry against MMP-3 shown the immunoreactivity was seen in hypertrophic chondrocytes in growth plate of 8 week-old rats (A and C). MMP-3 positive cells were increasing in CIA rats of 9 weeks of age (3 weeks after immunization) than that of normal rats (B and D, respectively).

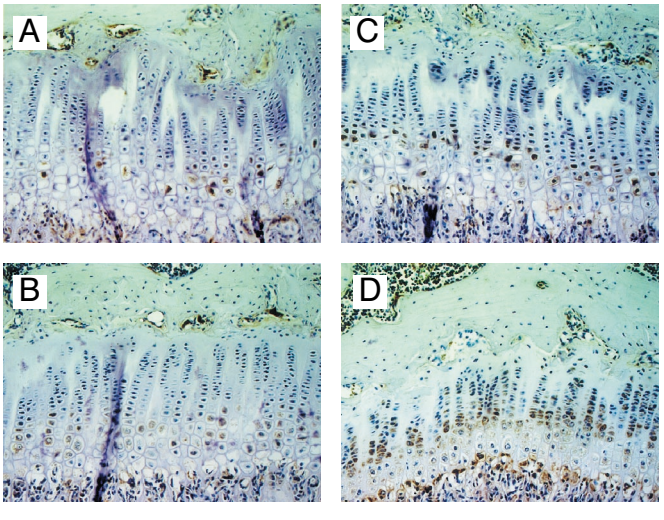


Figure 6. Growth plate of proximal tibia (immunohistochemistry against VEGF, 200x).
(A, B) Normal rats. (A, 8 weeks old; B, 9 weeks old).
(C, D) CIA rats. (C, 8 weeks old; D, 9 weeks old).
Immunohistochemistry against VEGF shown the immunoreactivity were seen in hypertrophic chondrocytes in growth plate of 8-week-old rats (A and C). Increase of VEGF-positive cells were seen in CIA rats of 9 weeks of age (3 weeks after immunization) than that of rats (B and D, respectively). Immunoreactivity against VEGF was also detected in swollen shaped chondrocytes in proliferating zone of CIA rats at 9 weeks of age.

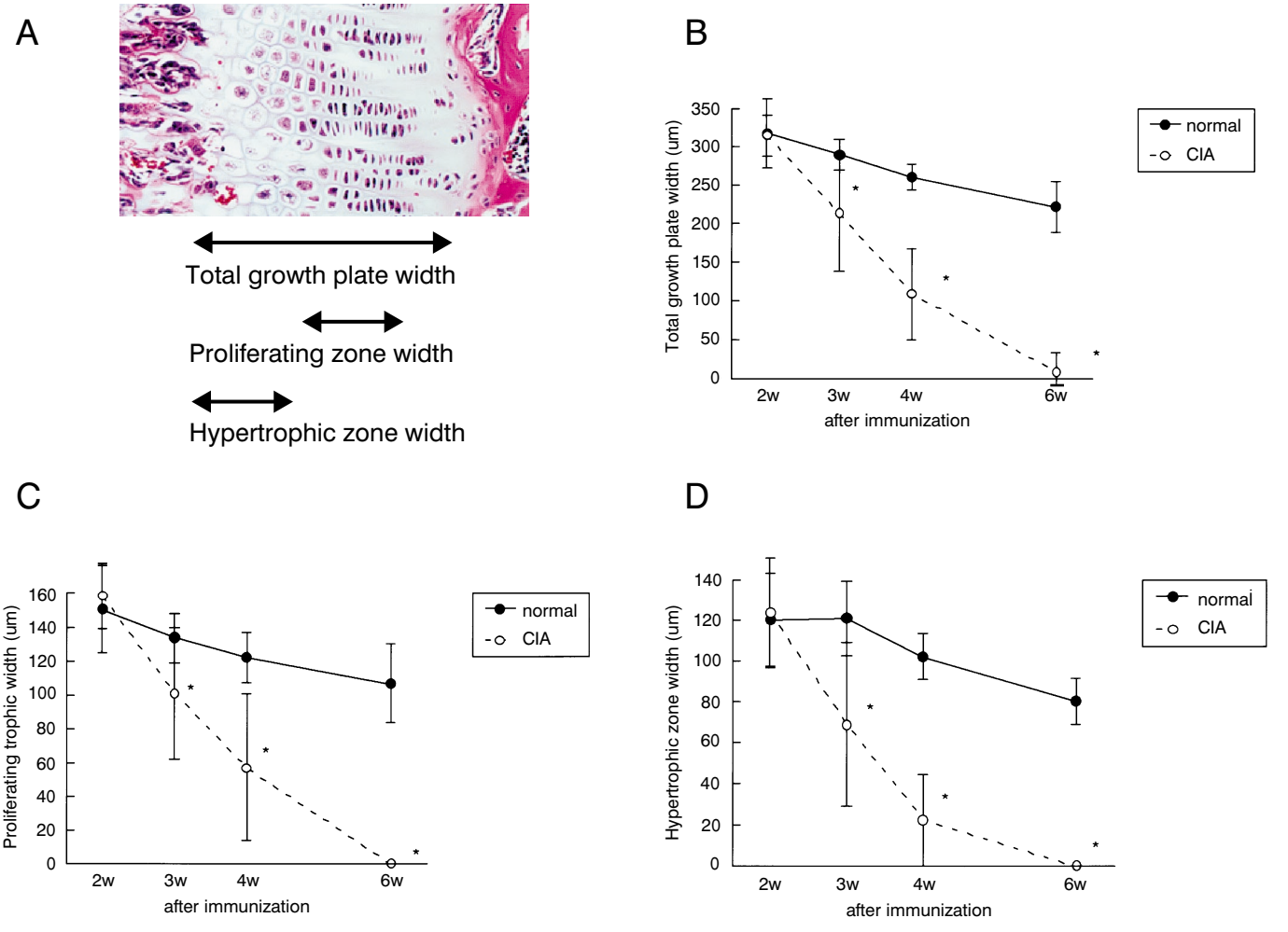


Figure 7. Growth plate widths at times following immunization.
(A) Total width, proliferating zone width, and hypertrophic zone width of growth plate in proximal tibia were measured.
(B) Total width of growth plate decreased more rapidly in CIA rats than in normal rats, and was greatly diminished 6 weeks after immunization (solid line, normal; dotted line, CIA).
(C, D) The decrease of hypertrophic zone width was more prominent than the decrease of proliferating zone width in CIA rats 3 and 4 weeks after immunization.

In this animal model of arthritis, it was clearly shown that such proinflammatory cytokines like TNF-α and IL-1β are involved in the pathogenesis of the disease, although there are a few reports concerning cytokine levels in the bone marrow in animals with CIA[20]. TNF-α and IL-1β are also implicated in the induction of MMPs, such as MMP-1 and MMP-3. These MMPs are involved in the degradation of the ECM[18,19,21-23]. The increase of MMP-3 producing cells in the growth plate of CIA might be the consequence of the greater production of proinflammatory cytokines in the bone marrow.

In addition, proteoglycan synthesis by chondrocytes could be affected by proinflammatory cytokines, which may lead to a decrease of proteoglycans in cartilage and decreased intensity of safranin-O staining.

During normal growth of the epiphyseal plate, stem cells initiate their programmed differentiation by frequent proliferation and typical columnar arrangement. Subsequently, cells cease proliferating and change morphometrically into their hypertrophic shape.

These hypertrophic chondrocytes mature further to produce matrix components. Thereafter matrix around the hypertrophic chondrocytes calcifies and the chondrocytes undergo apoptosis. These calcified matrices are replaced with trabecular bone by osteoclasts/chondroclasts and osteoblasts[12,25,26].

Various families of molecules have been identified as participating in the growth and development of bone[26-35]. Among these molecules, VEGF has been shown to be a key regulator of neoangiogenesis in cartilage growth plate, apoptosis of hypertrophic chondrocytes and osteoclasts recruitment into hypertrophic cartilage[36-38]. The present study demonstrates that the destruction of the growth plate in CIA

rats was accompanied with morphological changes of the hypertrophic chondrocytes and with an increase in the number of VEGF expressing cells. These findings suggest that over expressed VEGF in the growth plates of CIA rats might be involved with the abnormal ossification of the matrix and perhaps an increased recruitment of osteoclasts/chondroclasts, which resulted in destruction of the growth plate cartilage.

Many papers have assessed antiarthritic effectiveness of various molecules using the CIA model, however, the changes in growth plate cartilage have not been carefully evaluated. The quantitative analysis reported by Jee et al. showed a protective effect of methylpredonizolone against growth plate erosion in adjuvant-induced arthritis in rats[4]. This steroid is a well-known and potent anti-inflammatory agent that suppresses the production of inflammatory cytokines and MMPs[39,40]. These reports suggested that the protective effect of the steroid against growth plate erosion could be via suppression of some cytokines and MMPs, supporting some of the observations made in the present study. The detail of this process is still unclear and further investigation is necessary to elucidate the pathogenesis of growth plate destruction in inflammatory conditions. Clarification of the mechanism of this phenomenon could yield clinical benefits, especially in prevention of the premature closure of growth plate that is seen in juvenile rheumatoid arthritis and other diseases. This CIA rat model appears to be a useful model for further studies.

Acknowledgments

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