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The Protective Effect of Chondroitin from *Raja kenojei* Cartilage on Collagen-induced Arthritis in DBA/1J Mice

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Abstract In this study, we evaluated whether the oral administration of chondroitin from the cartilage of *Raja kenojei* is effective on the progression of rheumatoid arthritis (RA), using collagen-induced arthritic (CIA) mice. Arthritis development was delayed dose-dependently in the chondroitin-treated groups. The pre- and late-treated groups receiving 1,000 mg/kg of chondroitin had clinical scores that were reduced significantly by 56.9 ($p<0.05$) and 43.3% ($p<0.05$), respectively, compared to the vehicle-treated groups. Hematoxylin eosin staining and X-ray radiography showed that the chondroitins reduced the infiltration of inflammatory cells and prevented joint destruction of the knee and paw. Reverse transcription-polyerase chain reaction analysis revealed that chondroitin administration inhibited the expressions of tumor necrosis factor-α (TNF-α), interlukin-1β (IL-1β), and interferon-γ (IFN-γ) in joints more than the administration of vehicle. Chondroitin treatment also decreased the production of rheumatoid factors (RF), IgG and IgM, in the serum of CIA mice. These results indicate that chondroitin administration has a protective effect involving the inhibition of pro-inflammatory cytokine production in CIA mice.

Keywords: chondroitin, *Raja kenojei*, rheumatoid arthritis, collagen-induced arthritis, inflammation

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune syndrome that is characterized by chronic inflammation of the synovial tissue and destruction of cartilage and bone in the joints (1). *Raja kenojei* cartilage is reported to be exclusively composed of chondroitin sulfate (CS) covalently attached to a core protein, thus forming proteoglycans, which are embedded in a network of collagen fibrils (2). The solubilization of CS is required for the degradation of both the collagen matrix and the core protein (3). Many investigators have reported on the efficacies of chondroitin 4-sulfate (C4S) and chondroitin 6-sulfate (C6S) in experimental models, both *in vitro* and *in vivo* (4). However, the action of chondroitin from the cartilage of *R. kenojei* is not yet clear.

The chondroitin-induced arthritis (CIA) model has been used extensively to elucidate the pathogenic mechanisms that are relevant to human RA, and is widely used for the evaluation of potential anti-rheumatic agents (5). The acute stage of CIA is characterized by increased levels of mRNA for pro-inflammatory cytokines in the joints, such as tumor necrosis factor-α (TNF-α), interlukin-1β (IL-1β), and interferon-γ (IFN-γ) (6). Many of the major pro-inflammatory cytokines are produced in the rheumatoid synovium, and linked to a network or cascade with TNF-α at its apex (7). IL-1β was previously shown to be important in cartilage and bone destruction (8). This cytokine can be induced not only by many molecular signals such as IL-1, IFN-γ, and immune complexes, but also by cellular interactions in the rheumatoid synovium (9). Rhematoid facator (RF) is an antibody reactive against antigenic determinants on the Fc fragment of the IgG molecule. Approximately 80% of RA patients develop circulating RF antibodies (10). For this study, we report on the protective and therapeutic effects of chondroitin in CIA mice.

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Materials and Methods

Analysis of chondroitin disaccharides The cartilage of *R. kenojei* was dried with paper towels for 4 hr at room temperature, pulverized into small pieces using liquid nitrogen, and then extracted with 20 volumes of 4 M guanidine hydrochloride at 4°C with occasional stirring for 16 hr. The extracting solution was dialyzed against 6 M urea in Tris-HCl (Sigma, St. Louis, MO, USA) at pH 8.1 overnight at 4°C. The resultant dialysate was filtrated through 0.45 μm membrane filter (Millipore, Billerica, MA, USA) and concentrated by Amicon Ultra PL 10 (Amicon, Beverly, MA, USA). The crude solutions were eluted on a Sephadex G-50 column (2.5×20 cm; Sigma) equilibrated with 6 M urea in Tris-HCl (Sigma) at pH 8.1 at a flow rate of 1.0 mL/min. The A_{280} of each fraction was monitored, and the active fractions were pooled and concentrated. The sulfated glycosaminoglycan (GAG) was quantitatively measured by dimethylmethylene blue (DMB) method as described by Whitley *et al.* (11). We

formed unsaturated chondroitin disaccharides via alkaline borohydride treatment and chondroitinase A, B, and C digestion. High performance liquid chromatography (HPLC) was performed with YMC-Pack NH_2 column (250×4.6 mm, 5 μm) equilibrated with 10 mM ammonium phosphate buffer (pH 6.7) at a flow rate of 1.0 mL/min at 37°C. The column was eluted with 10 mM ammonium phosphate buffer (pH 6.7) for 10 min, followed by a linear gradient of 10-500 mM ammonium phosphate (pH 6.7) for 20 min, and 500 mM ammonium phosphate (pH 6.7) for 10 min; the eluate was monitored at 232 nm. Authentic Δdi-6S and Δdi-4S, prepared from the shark cartilage, were used as standards. Individual peaks were also purified and analyzed by matrix-assist laser desorption ionization time-of-flight/mass spectrometry (MALDI-TOF/MS) (12).

Preparation of chondroitins The cartilage of *R. kenojei* was treated with 3 N HCl to eliminate undesired materials. After 3 hr, the cartilage was rinsed with distilled water three times, and then melted at 70°C overnight. The supernatant was obtained by centrifugation at 3,000×g for 10 min and the GAG content was measured by DMB assay. The chondroitin concentrations were adjusted to 100, 500, and 1,000 mg/kg, respectively.

The CIA model The DBA/1J mice used in this study were 7-8 week-old males purchased from Japan SLC, Inc. (Shizoka, Japan) The animals were fed standard rodent chow (Harlan, Indianapolis, IN, USA) and water *ad libitum* at a temperature of 22±2°C and a relative humidity of 50±5%, and were maintained in a 12 hr light/dark cycle under specific pathogen-free conditions according to the institutional instructions of Chonnam National University, Korea (13). Chick collagen (Sigma) was dissolved in 10 mM acetic acid at a concentration of 2 mg/mL by stirring overnight at 4°C. The collagen was emulsified with an equal volume of complete Freund's adjuvant (Chondrex Inc., Redmond, WA, USA) containing 5 mg/mL of *Mycobacterium tuberculosis*. The first immunization was performed with 100 μL of emulsion by hypodermic injection at the tail base on day 1. A boost injection was administered on day 21; however, the collagen was emulsified using incomplete Freund's adjuvant and the injection site was proximal to the first immunization.

Experimental groups The prepared chondroitin was dissolved in distilled water and administered daily, at a volume of 200 μL/head. The administration schedule is described in Scheme 1. To evaluate the protective and therapeutic effects of the chondroitin on CIA, the mice were subdivided into non-treated and pre-treated groups that received orally administered chondroitin daily at 100, 500, and 1,000 mg/kg, or the vehicle, for 31 days (10 days before the primary immunization to day 21); the late-treated groups were treated with the same amounts of chondroitin or vehicle for 20 days after the boost injection.

Clinical assessment of arthritis The clinical signs of arthritis were assessed in each of the 4 limbs and recorded 3 times weekly. Briefly, the following scoring system was used: 0 = normal (no signs of arthritis); 1 = swelling and/or redness of 1 to 2 interphalangeal (IP) joints; 2 = the involvement of 3 to 4 IP joints or 1 larger joint; 3 = more than 4 joints were red/swollen; 4 = severe swelling of the entire paw. The total score for clinical assessment was based on all 4 paws, with a maximum possible score of 16 for each mouse. Scoring was conducted under blinded conditions.

Histopathological and radiographic assessment The mice were sacrificed on day 41, and the paw and knee joints were stained with hematoxylin eosin for histological examination under a light microscope (13). The paws and knees were radiographed using an X-ray machine (Hitex Co., Ltd., Kanagawa, Japan) with 40 kW expositions for 10 sec. Each limb was assessed for the presence of soft tissue swelling and joint destruction.

Reverse transcription (RT) and polymerase chain reaction (PCR) The knees joints were homogenized and the total RNA was extracted with RNeasy mini kit (Qiagen, Valencia, CA, USA). The total RNA (1 μg) was reveres-transcribed and cDNA amplification was performed with RT premix (Bioneer, Daejeon, Korea). To evaluate the expression levels of pro-inflammatory cytokines, primers for TNF-α, IL-1β, and IFN-γ were used in RT-PCR, and are summarized in Table 1. Amplifications were performed with an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, with a final extension step at 72°C for 5 min.

Evaluation of RF Blood was drawn from the mice that

Table 1. Primers used for reverse transcription PCR

Accession No.	Gene	Primer sequence	Expected size
NM_001101	β-Actin	5′-AGCCATGTACGTAGCCATCC-3′	250 bp
		5′-TTTGATGTCACGCACGATTT-3′	
NM_013693	TNF-α	5′-CTCAAATGGGCTTTCCGAATT-3′	101 bp
		5′-TCCAGCCTCATTCTGAGACAGA-3′	
NM_008361	IL-1β	5′-CTTCCCCAGGGCATGTGA-3′	101 bp
		5′-ACCCTGAGCGACCTGTCTTG-3′	
NM_008337	IFN-γ	5′-TGCTGATGGGAGGAGATGTCT-3′	101 bp
		5′-TTTCTTTCAGGGACAGCCTGTT-3′	

were sacrificed on day 41; it was then centrifuged at 10,000×g for 20 min at 4°C and the serum was collected. The levels of mouse RF IgG and IgM were measured using ELISA kit according to the manufacturer's protocol (Shibayagi Co., Ltd., Shibikawa, Japan).

Statistical analysis The data are presented as the mean ± standard error (SE). The results were compared using Student's *t*-tests.

Results and Discussion

C4S and C6S as major chondroitin components of *R. kenojei* cartilage The cartilage of *R. kenojei* was extracted as described in the Materials and Methods. The yield of proteoglycan was approximately 1.0 mg/mL. The overall proteoglycan yields were near 85%, and they were recovered in the major peak containing fractions 7 to 24 (Fig. 1a). The yielded pools were analyzed on an YMC-Pack NH_2 column as described above. Two main peaks were obtained from enzyme digest, and Peak 1 and 2 could be assigned to UA-GalNAc-6S and UA-GalNAc-4S, respectively (Fig. 1b). Three additional minor peaks showed different retention patterns, and were considered GAG derivatives. These data indicate that the main types of chondroitin sulfate from *R. kenojei* cartilage are C6S and C4S. After HPLC analysis, the peaks were pooled and compared with chondroitin sulfate disaccharide standards (Δdi-6S and Δdi-4S) to identify the ionization of molecular mass using MALDI-TOF/MS. The MALDI-TOF spectra and the peak at *m/z* 458 in the negative ion spectrum that corresponded to the presence of M-2Na£´H, and that at *m/z* 480 corresponding to M-Na, indicated the presence of single-charged disaccharides, with the exception of DHB matrix (Fig. 1c and 1d). These results indicate that Peak 1 and 2 correspond to UA-GalNAc-6S and UA-GalNAc-4S, respectively.

As mentioned above, C4S and C6S are the major chondroitin components (Fig. 1). CS is produced by chondrocytes found primarily in cartilage and major connective tissues. It belongs to a family of hetero-polysaccharides called GAGs, which comprise the ground substance in the extracellular matrix of connective tissues (14). The action of orally administered CS has not been clarified, but several possible actions such as the maintenance of the structure and function of cartilage (15), pain relief of osteoarthritic joints, and anti-inflammatory activities (16), have been proposed. Furthermore, C4S possesses antioxidant activity that may reduce free radicals and inhibit lipid peroxidation (4). Finally, the use of these molecules as therapeutic agents has shown positive outcomes both in humans and experimental animal models (4, 17).

Effect of chondroitins on clinical scores Two days after booster injection, joint swelling appeared in one or more of the hind paws of the mice in the vehicle-treated groups. However, the progression of arthritis was delayed dose-dependently in the chondroitin pre- and late-treated groups of mice. Among them, the pre- and late-treated groups receiving 1,000 mg/kg of chondroitin significantly reduced their clinical scores by 56.9% ($p<0.05$) and 43.3%

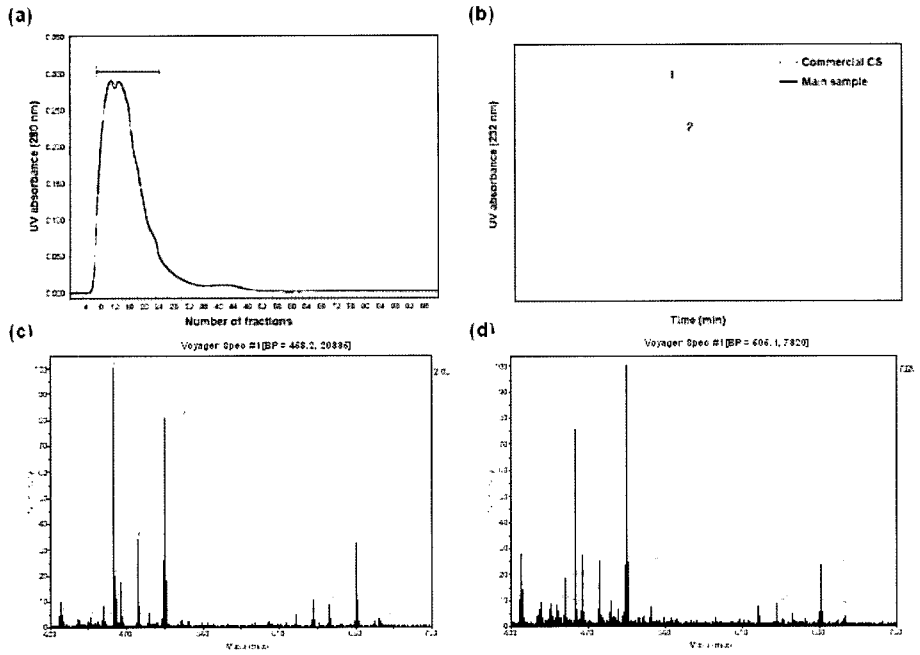

Fig. 1. Analysis of chondroitin sulfate from the cartilage of *R. kenogei*. (a) The elution profile of proteoglycan from the cartilage of *R. kenojei* by Sephadex G-50 column chromatography; (b) The HPLC chromatogram showed that the retention time of the commercial CS and main sample are identical. Peak 1 and 2 correspond to UA-GalNAc-6S (C6S) and UA-GalNAc-4S (C4S), respectively; (c) The MALDI-TOF spectrum of peak 1 fractionated by HPLC; (d) The MALDI-TOF spectrum of peak 2 fractionated by HPLC. The ion at *m/z* 458 is the sulfated disaccharide products.

($p<0.05$), respectively, compared to the vehicle-treated groups (Fig. 2). These data suggest that pre-treatments of chondroitin may be more effective than late-treatments on arthritis development.

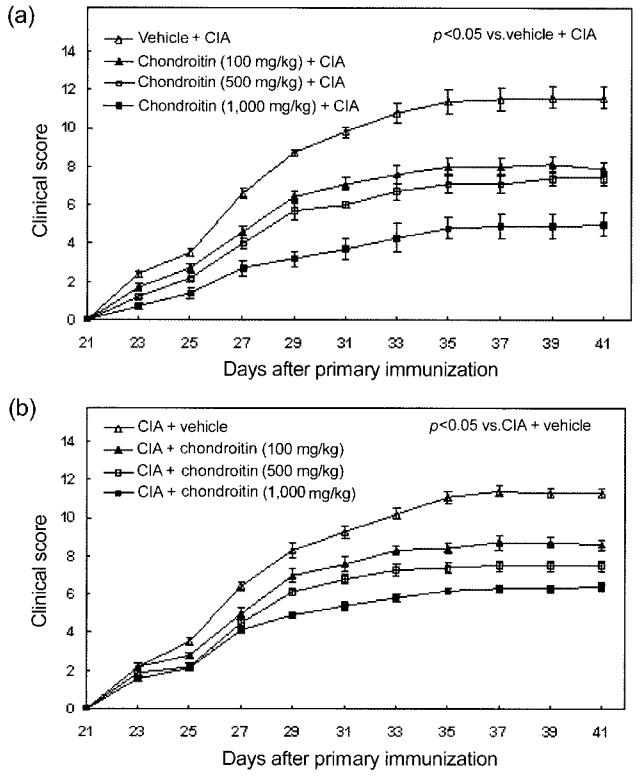

Fig. 2. Clinical scores in CIA mice. Clinical assessment was performed 3 times weekly from day 21 to day 41 using a 4-point scale (0-4) for each paw. The total score for clinical assessment was measured on 4 paws per mouse (maximum score of 16). The data represent the mean±SE of 10 mice in each group. (a) The protective effects of chondroitin in CIA mice. (b) The therapeutic effects of chondroitin in CIA mice.

We found that chondroitin administration clearly inhibited arthritis progression by decreasing clinical disease activity and swelling (Fig. 2). These results offer supportive evidence that chondroitins have potential protective effects against arthritis and joint injury.

Histological differences and radiologic findings in CIA mice The joint sections of the untreated DBA/1J mice showed no synovial thickening or inflammatory cell infiltration, and the joint structures were well maintained (Fig. 3a). In contrast, the tissue sections from the CIA mice pre- or late-treated with vehicle, showed synovial hyperplasia and the infiltration of mononuclear cells, which is characteristic of CIA (Fig. 3b). Interestingly, inflammatory cell infiltration and synovial cell hyperplasia decreased in the chondroitin-treated groups (Fig. 3c and 3d). These suppressive effects on CIA were marked, especially in the chondroitin pre-treated groups (Fig. 3c). Also, from a radiographic point of view, there was no evidence of joint deformity or soft tissue swelling in the DBA/1J mice (Fig. 3e). As expected, severe joint destruction along with soft tissue swelling were observed in the CIA mice pre-treated with vehicle (Fig. 3f). Whereas, both the chondroitin pre-treated groups (Fig. 3g) and late-treated groups (Fig. 3h) had relatively normal joint structures with little soft tissue swelling.

In the histopathologic and radiographic evaluations, chondroitin remarkably inhibited inflammatory cell infiltration and prevented joint deformity in the CIA mice (Fig. 3). It is well known that chronic synovial inflammation and joint destruction play major roles in the clinical outcomes of RA (1, 18, 19). These findings provide support that chondroitins have protective and therapeutic effects in CIA mice by attenuating the development of arthritis and joint injury.

Effects of chondroitin on pro-inflammatory cytokine expression and the production of rheumatoid factors Using clinical assessments, immunohistochemistry, and X-ray radiography, we found that arthritis development was

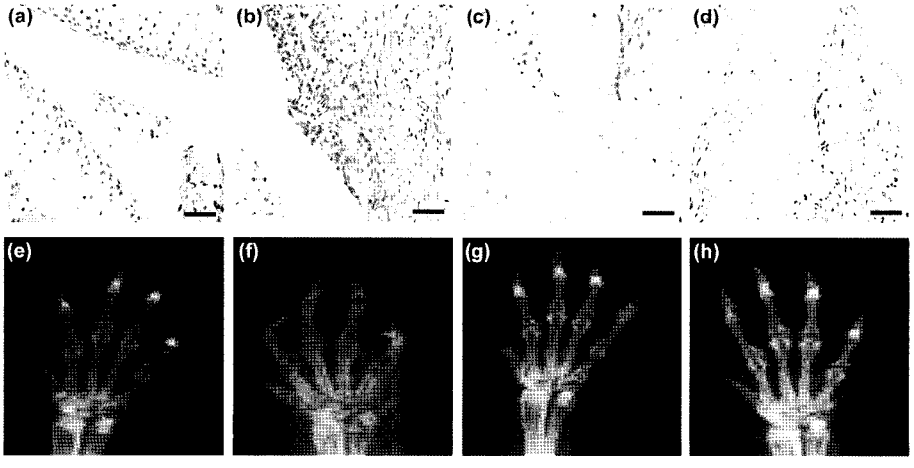

Fig. 3. Histopathologic and radiologic analyses. CIA mice were treated with vehicle or chondroitin as described previously. Representative tissue stains from 1 of 3 independent experiments are shown. (a, e) Normal (non-treated); (b, f) vehicle + CIA; (c, g) chondroitin (1,000 mg/kg) + CIA; (d, h) CIA + chondroitin (1,000 mg/kg). Bars indicate 100 μm.

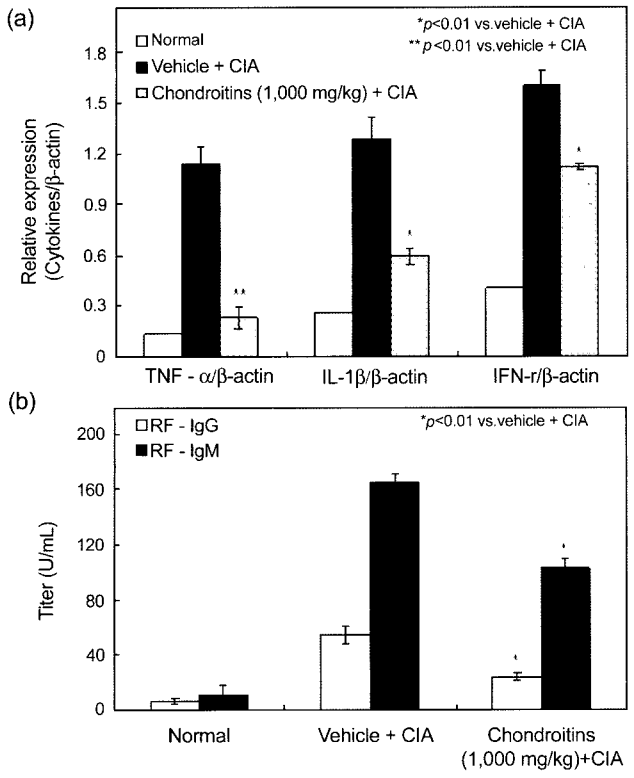

Fig. 4. The expression of pro-inflammatory cytokines and rheumatoid factor production. (a) The expressions of pro-inflammatory cytokines were determined by RT-PCR. The expression of β-actin was monitored as a control. The data values are mean± SE for 2 animals from each group. (b) The levels of RF-IgG and -IgM were determined by ELISA in chondroitin-treated mice. The data represent the mean±SE of least 2 mice in each group.

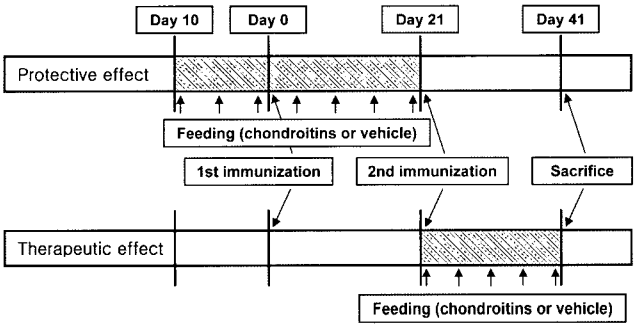

Fig. 5. Administration schedules.

retarded dose-dependently in the joints of CIA mice for the chondroitin-treated groups. Furthermore, to determine whether the protective effect of chondroitin at high doses was related to anti-inflammation in the CIA mice, expressions of the pro-inflammatory cytokines TNF-α, IL-1β, and IFN-γ were analyzed in the joints of the chondroitin pre-treated groups. The expression levels of TNF-α, IL-1β, and IFN-α were increased in the vehicle-treated groups by approximately 8.3-, 5.0-, and 4.0-fold, respectively, compared to the normal mice. However, TNF-α, IL-1β, and IFN-α transcription were lowered by 80 ($p<0.01$), 54 ($p<0.05$), and 30% ($p<0.05$), respectively, in the groups treated with 1,000 mg/kg of chondroitin compared to the vehicle-treated groups (Fig. 4a). We also analyzed RF-Ig levels in the serum of mice that received chondroitin pre-treatments of 1,000 mg/kg. In the normal mice, the levels of the RF antibodies, IgG and IgM, were less than 10 U/mL. However, RF-IgG and -IgM levels were increased 9.1- and 15.2-fold in the vehicle-treated CIA mice compared to the normal mice. The RF-IgG and -IgM levels of the chondroitin pre-treated mice were significantly reduced, approximately 60 ($p<0.01$) and 40% ($p<0.01$) respectively, versus the vehicle-treated groups (Fig. 4b).

Pro-inflammatory cytokines, including TNF-α and IL-1β, have been investigated intensively for their roles in the pathogenesis of CIA. It is well known that they play crucial roles in joint destruction in CIA (20, 21). Earlier studies have shown that auto-reactive T-cells may play an important role in the development and pathogenesis of autoimmune arthritis (1). The response of T-cells to unknown antigens can trigger T-cell activation and an inflammatory cascade involving T-cells, macrophages/monocytes, B-cells, and activated synoviocytes in CIA. A variety of infiltrating immune cells and resident synoviocytes can produce a complex array of cytokines and other soluble mediators that are thought to be responsible for cartilage destruction and bony erosion (21).

In our study, chondroitins markedly reduced inflammatory cell infiltration and inhibited the expressions of TNF-α, IL-1β, and IFN-γ. Therefore, we propose that the effects of chondroitins, such as anti-inflammatory and cartilage/bone-protective effects, may be mediated partly through the inhibition of pro-inflammatory cytokine expression.

B-Cells have the potential to play critical roles in the pathogenesis of RA (10). For example, they can promote the *in situ* activation of tissue-infiltrating T-cells in patients with rheumatoid synovitis (22). Synovial B-cells that are activated by autoreactive T-cells are cellular sources of RF, which is a serologic marker for RA and has pathogenic roles in the disease (10). To investigate whether RF production is affected by chondroitins, we determined the levels of RF-IgG and -IgM in CIA mice. Our data show that the levels of RF-IgG and -IgM in the chondroitin-treated mice were significantly lower than the vehicle-treated mice (Fig. 4b). Thus, the inhibitory effect of chondroitin on the production of RF-IgG and -IgM is partly responsible for the attenuated CIA.

In conclusion, we demonstrated that chondroitins reduced paw swelling, clinical indices, histopathologic/radiographic severity, the expression of pro-inflammatory cytokines, and RF antibody production. Our results suggest that chondroitins have protective effects involving joint protection and anti-inflammatory properties through the inhibition of pro-inflammatory cytokine expression in chronic inflammatory joint diseases. Finally, the protective effects of chondroitin on CIA may provide new insights for its pharmacological functions.

Acknowledgments

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