Effects of Low Molecular Weight Chondroitin Sulfate on Type II Collagen-Induced Arthritis in DBA/1J Mice

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In order to evaluate the improvement in the treatment of chronic arthritis, we investigated chondroitin sulfate (CS) in vitro and in vivo. LMWCS was prepared by a chemical depolymerization product induced by hydrogen peroxide in the presence of copper salts. LMWCS (300 mg/kg) and CS (1200 mg/kg) were orally administered to DBA/1J mice once daily for 14 d prior to initial immunization with type II collagen. Their elastase activities and the production of cytokines in sera were examined on type II collagen-induced arthritis in DBA/1J mice. We also compared the paracellular transport of LMWCS and CS across Caco-2 cell monolayers and examined the inhibitory effects on elastase activities. LMWCS inhibited elastase activity slightly, but CS did not show inhibition. Hind paw edema was significantly decreased by LMWCS treatment. Levels of anti-type II collagen antibody and tumor necrosis factor-alpha (TNF-α) in sera were also reduced by LMWCS treatment but not in case of CS, although no significant difference was observed between LMWCS and CS on interleukin-6 (IL-6) induction. The LMWCS preparation showed preventive effects on the type II collagen-induced arthritis in DBA/1J mice and better permeability through Caco-2 cells.

Key words chondroitin sulfate; collagen-induced arthritis; Caco-2 cell

Chondroitin sulfate (CS) is a glycosaminoglycan, which is composed of an alternating sequence of sulfated and/or unsulfated residue of d-glucuronic acid (GlcA) and d-N-acetylgalactosamine (GalNAc) linked by β(1→3) and β(1→4) bonds.1) CS is a major class of glycosaminoglycans required for the formation of proteoglycans found in the joint cartilage. It has been well established that basic damage to the arthritic cartilage involves the alteration of proteoglycans and collagen fibers.2,3)

The therapeutic uses of intact glycosaminoglycans, their low molecular mass derivatives and mixtures of different percentages of heteropolysaccharides have been markedly increased by the knowledge of their pharmacological properties and biological functions.3,4) The use of glycosaminoglycans, is of particular interest, as these compounds have been shown to stimulate the chondrocyte synthesis of proteoglycans, especially in aggrecans. CS may be employed as a chondroprotective drug5) and administered in the therapy of osteoarthritis of the knee6) by intramuscular and oral routes. The oral route of CS as a chondroprotective drug is especially attractive, because it allows simplified use, which is more compatible with long term administration.

Although it may be difficult that molecules with high molecular mass and charge density pass through the gastrointestinal tract and its physicochemical properties.7–10) Furthermore, glycosaminoglycans with a low molecular mass and charge density are preferentially absorbed.8,11) In case of CS, some reports have been issued concerning its oral administration, metabolisms and chondroprotective function in arthritis.5,12–14)

However, because of its high molecular weight and charge density, CS absorption seems to be restricted to the gastrointestinal tract.

In the present study, CS was chemically depolymerized to promote absorption through the gastrointestinal tract and its pharmacological activity in an experimental animal model of type II collagen-induced arthritis was compared with CS. The absorption of LMWCS was evaluated using the Caco-2 cell monolayer system, which may well represent the in vivo maturation of the intestinal epithelium.

MATERIALS AND METHODS

Materials CS, originating from the bovine trachea, was purchased by New Zealand Pharmaceuticals. Human leukocyte elastase [EC. 3.4.21.37], type II collagen from chicken sternal cartilage, complete Freund’s adjuvant and alkaline phosphatase substrate (p-nitrophenyl phosphate disodium), CS disaccharides (Δdi-0S, Δdi-4S, and Δdi-6S), and a chromogenic substrate, N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide were obtained from Sigma (St Louis, MO, U.S.A.). Alkaline phosphatase-conjugated anti-mouse immunoglobulin G antibody was purchased from Calbiochem (La Jolla, CA, U.S.A.). The enzyme-linked immunosorbent assay (ELISA) kits for murine TNF-α and IL-6 were from R&D Systems (Minneapolis, MN, U.S.A.), other reagents and chemicals were of the best grade available.

Animals Male DBA/1J mice, aged 5—6 weeks, were purchased from Japan SLC Inc. (Shizuoka, Japan) and acclimatized to standard laboratory conditions (25±3 °C, 55±5% humidity and 12 h light/dark cycle) for 7 d. All animal works were carried out in a specific pathogen-free barrier zone at

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 Seoul National University Hospital in accordance with the procedure outlined in the Guide for the Care and Use of Laboratory Animals.

Preparation of Low Molecular Weight Chondroitin Sulfate  LMWCS was obtained by using a controlled chemical depolymerization process induced by free radicals from hydrogen peroxide in the presence of copper salts.  The average molecular weight of LMWCS was characterized by high-performance size-exclusion chromatography (HPSEC) using a TSK-G3000SW column (4.6×300 mm, Tosoh, Japan) equilibrated with 100 mM NaCl at a flow rate of 1.0 ml/min. The standards used for the calibration of the column were two types of CS A of known molecular weights (MW=40000 and 15000) and a chondroitin sulfate-derived oligosaccharides (n=5, MW=2265; n=10, MW=4530; n=15, MW=6795).  Structures of these species were confirmed by 1H-NMR (nuclear magnetic resonance) spectroscopy as previously described.

Compositional Analysis of LMWCS and CS Each 500 μg of samples dissolved in 900 μl of 50 mM sodium phosphate buffer (pH 7.0) were treated with 100 mU of Bacteroidal chondroitinase ABC at 37 °C for 24 h.  After heating for 5 min and filtering on Millipore filter (0.45 μm), the digestion mixtures were analyzed and calculated based on the calibration curves of each authentic unsaturated CS disaccharide.  High-performance liquid chromatography (HPLC) was performed on a 5 μm particle size strong-anion exchange (SAX) analytical column from Thermo Hypersil-Keystone (Bellefonte, PA, U.S.A.) of dimension 0.46×25 cm using ÄKTA purifier controlled by UNICORN software 3.1 from Amersham Pharmacia (Uppsala, Sweden) as previously described.

Effects on the Inhibition of Elastase Activity  This assay was essentially performed according to a previous report with some modifications.  Briefly, each sample (100 μg) containing LMWCS and CS was dissolved in 455 μl of 60 mM Tris–HCl containing 5% DMSO (pH 7.5).  Human leukocyte elastase (20 μl, 1 mU/μl) dissolved in the same buffer was added and the mixture was incubated for 3 min at 37 °C.  The chromogenic substrate dissolved in the same buffer (125 μl, 1.4 mM) was added and the mixture was incubated for 1 h at 37 °C.  The reaction was stopped by adding 100 μl of 0.5 M acetic acid and the absorbance was measured at 405 nm.  The percent inhibition of elastase activity was calculated as residual activity versus a control and elastatin was used as a postive control.

Induction of Arthritis  Type II collagen was dissolved overnight at 4 °C in 50 mM acetic acid to 2 mg/ml.  This solution was then emulsified in an equal volume of complete Freund’s adjuvant in an ice-cold water bath.  Arthritis was induced by the intradermal injection of 0.1 ml of this emulsion into the base of the tail.  Mice were boosted using the same schedule 21 d later.

Treatment of Samples  LMWCS and CS were orally administered once daily for 14 d prior to initial immunization with type II collagen at doses of 300 and 1200 mg/kg, respectively.  The arthritic control group received an equivalent volume (0.3 ml) of physiological saline based on body mass according to the same schedule.

Measurement of Hind Paw Edema  Edema in the hind paw was measured before the initial injection of type II collagen and after the booster injection, using a digital gauge.  The mean thickness of the right and left hind paws was recorded as paw thickness over a period of 49 d.

Preparation of Serum  Blood was collected by heart puncture.  After clotting at room temperature, the blood was kept overnight at 4 °C and the serum was collected by centrifuging at 2000×g for 15 min.  All samples were stored at −80 °C until required.

Measurement of Anti-type II Collagen Antibody Titers  Anti-type II collagen antibody titer was determined as previously described.  In brief, a 96-well ELISA plate was coated with 10 μg/ml bovine type II collagen in 50 mM sodium carbonate buffer, pH 9.8 containing 5 mM MgCl2 (100 μl/well), incubated at 37 °C for 1 h and then left overnight at 4 °C.  After washing with phosphate buffered saline (PBS)–Twee 20 (0.05%) three times, the plates were blocked with PBS containing 1% bovine serum albumin at 25 °C for 1 h.  Sera diluted 1:10000 in PBS–Twee 20 were added to 96-well plates coated with type II collagen and incubated overnight at 4 °C.  After washing with PBS–Twee three times, alkaline phosphatase-conjugated anti-mouse immunoglobulin G antibody diluted at 1:10000 in PBS–Twee 20 was added to each well and the plate was incubated at 37 °C for 1 h.  The plate was then rewashed and the substrate (p-nitrophenyl phosphate disodium, 10 mg/ml in 50 mM sodium carbonate buffer, pH 9.8, containing 5 mM MgCl2) was added.  The absorbance was measured at 405 nm with an E-max microplate reader (Molecular Device, Sunnyvale, CA, U.S.A.).

Effects on the Production of Cytokines in Serum  Cytokine levels in serum were determined using enzyme-linked immunosorbaent assay (ELISA) kits for murine TNF-α and IL-6 (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer’s instructions at room temperature.

Assessment of Permeability through Caco-2 Monolayers  The maintenance of integrity of Caco-2 cell monolayers and experiments were performed as previously described.  The amount of LMWCS and CS transported across Caco-2 cells was determined using the dimethyl-methylene blue assay.

Data Analysis  Data were expressed as means±S.E.M.  To analyze the data statistically, we performed one-way analysis of variance (ANOVA) for repeated measurements of the same variable, and used Duncan’s multiple range t-test to determine which means were significantly different from that of the control.  We considered differences significant at p<0.01 and p<0.05.

RESULTS

Preparation of Low Molecular Weight Chondroitin Sulfate  The average molecular weight of LMWCS was determined as 3000 Da by gel-filtration chromatography (Fig. 1).  The major repeating structure of LMWCS was confirmed by 1H-NMR as shown in Fig. 2.  The reporter groups of LMWCS in their chemical shifts were consistent with those of CS.

Disaccharide Analysis of LMWCS  HPLC profiles and % composition of disaccharides are shown in Fig. 3.  Both chromatograms show similar profiles and the content of disaccharides of samples are essentially consistent.  The results
suggest that chemical depolymerization of CS does not affect the disaccharide composition.

**Effects on Elastatse Activity** LMWCS significantly inhibited the elastase activity at the same concentration, but the intact CS did not inhibit it, unlike that reported previously\(^{22}\) and no difference was found between the control and CS as shown in Fig. 4.

**Induction of Arthritis and Measurement of Hind Paw Edema** After a booster injection of type II collagen was administered on day 21, hind paw thickness peaked on day 35 and then gradually subsided (Fig. 5). Although statistical significance was not observed, the treatment of LMWCS significantly reduced hind paw thickness compared to that of chondroitin sulfate.

**Measurement of Anti-type II Collagen Antibody Titers** On day 49, the levels of anti-type II collagen antibodies were measured, and were found to be higher in arthritic control mice than in the normal control. LMWCS moderately re-
duced the level in the anti-type II collagen antibody titer (Table 1). This result is consistent with the finding of reduced hind paw thickness.

Effects on the Production of Cytokines in Serum In DBA/1J mice, LMWCS suppressed TNF-α production in serum, but the intact CS did not influence TNF-α serum levels (Fig. 6A). In contrast, both CS and LMWCS decreased IL-6 levels in serum compared to the arthritis control, as shown in Fig. 6B.

Measurement of Transport Amount Across Caco-2 Cell Monolayers After treating each sample on the apical side, the transport of LMWCS across Caco-2 monolayers was significantly increased compared to that of CS, as shown in Fig. 7. After 3 h of treatment, 3% of LMWCS of loading amount was detected in the basolateral side. But the transport amount of CS in the basolateral side could not be detected throughout the period of incubation.

DISCUSSION

Connective tissues are primarily comprised of collagen and proteoglycans. Aggrecan, a large aggregated CS proteoglycan, is present at high concentration in cartilage and provides this tissue with many of its characteristic physicochemical properties. In normal articular cartilage, CS is produced by chondrocytes and synoviocytes from the precursor amino sugar, glucosamine. Once excreted into the connective tissue matrix, these compounds spontaneously assemble into proteoglycan aggregates. Although controversial results on the intestinal absorption of CS have been reported, we were able to demonstrate that the transport of CS via the intestinal epithelium is very restricted by Caco-2 cell monolayers. In contrast, the depolymerization product, LMWCS could traverse the membrane gradually, although their high molecular weights and negative charges may restrict in the intestinal absorption.

Neutrophil elastase, a granule serine protease, is a member of the proteinase family, which hydrolytically degrade connective components, such as elastin, proteoglycan, fibrinectin, and collagen types I, II, III and IV. Because elastase can readily degrade matrix proteins, the inhibitors are likely the therapeutic advantage in these pathogenic conditions of arthritis. Volpi reported that glycosaminoglycans, such as CS and heparin, have the ability to inhibit the leukocyte elastase activity because of their charge density. Unlike previous reports, we found that LMWCS is superior to CS in terms of inhibiting elastase activity in vitro when applied at the same dose (Fig. 3). Further study is needed to clarify the relationship between charge density and inhibitory activity.

Collagen-induced arthritis has been used as a rheumatoid arthritis model and the role of collagen as an autoantigen in rheumatoid arthritis has been extensively studied. In the present study, the antibody level to type II collagen was moderately low as compared to the other groups, when LMWCS was orally administered. Although the etiology of rheumatoid arthritis remains unknown, the excessive production of proinflammatory cytokines, including TNF-α, IL-1 and IL-6, has been implicated as a major factor in the pathogenesis of the diseases. Moreover, both TNF-α and IL-1 have been detected in the joints of rheumatoid arthritis patients and are known to possess properties, which are consistent with a pathogenic role. TNF-α and IL-1 seem to function synergistically to induce the synthesis and secretion of IL-6 as effectors. It has been well demonstrated that TNF-α, IL-1 and IL-6 are highly expressed at sites of diseases in collagen-induced
Thus, the regulation of these cytokines in synoviocytes or the synovial environment may be important in the pathogenesis and therapy of rheumatoid arthritis. As stated above, we found that the LMWCS was associated with decreased serum levels of TNF-α, IL-6 and anti-collagen antibody compared with intact CS or arthritis control. The moderate decrease of serum level of TNF-α may be due to a low level of LMWCS, even though it is absorbed in vitro gradually. But it can significantly decrease hind paw edema, which can be related to the reduction of these cytokine levels in serum and the inhibition of the elastase activity.

In conclusion, the effect of LMWCS on collagen-induced arthritis was superior to that of intact CS, which might be due to better absorption via the gastrointestinal tract and regulation in inflammatory responses. Our study suggests that LMWCS may be useful in prevention of rheumatoid arthritis owing to chronic inflammation.

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