Chondroitin-4-sulphate inhibits NF-kB translocation and caspase activation in collagen-induced arthritis in mice


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Summary

Objective: Free radical damage, inflammation, and apoptosis play a critical role in the onset and progression of cartilage erosion in arthritis. Many studies have demonstrated that glycosaminoglycans (GAGs), and chondroitin-4-sulphate (C4S) in particular, possess antioxidant activity that is able to inhibit lipid peroxidation which is the main mechanism of free radical-mediated biological injury. In addition to the effect directly exerted by reactive oxygen species (ROS), the activation of nuclear factor kβ (NF-kβ) and caspases may contribute substantially to increase inflammation and cell damage. We studied whether the antioxidant action of chronic C4S treatment to reduce ROS injury involves NF-kβ and caspases modulation using an experimental model of collagen-induced arthritis in mice.

Methods: Arthritis was induced in mice via an intradermal injection at the base of the tail of 100 μl of emulsion containing bovine type II collagen in complete Freund’s adjuvant.

Results: Arthritis provoked the following: severe oedema and inflammation in the hind paws; lipid peroxidation in the joints [measured by 8-isoprostane (8-IPE) levels]; reduction of the endogenous antioxidants catalase (CAT) activity and reduced glutathione (GSH) levels; induction of NF-kβ translocation; a loss of cytoplasmic NF-kβ inhibitor alpha (IkBα); an increase in metalloproteinase-13 (MMP-13), caspase-3 and caspase-7 gene expression and their related protein; the induction of cartilage polymorphonuclear (PMN) activation and infiltration [evaluated by elastase (ELA) assay] and cartilage alterations evaluated by histological analysis. Intraperitoneal administration of different doses of C4S (for 25 days), ameliorated all the symptoms of inflammation in the articular knee and paw joints, limited lipid peroxidation, inhibited NF-kβ activation and IkBα protein loss, decreased mRNA MMP-13 and caspases expression and their related protein, restored endogenous antioxidants, and reduced PMN accumulation in the damaged cartilage.

Conclusion: The evidence that C4S was able to inhibit NF-kβ and apoptosis activation supports the hypothesis that the C4S effect depends on reduction of ROS production, although other direct effects cannot be excluded.

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Key words: Chondroitin sulphate, NF-kβ, Caspases, Arthritis, Antioxidants, Lipid peroxidation.

Introduction

Arthritis is an autoimmune disease of unknown aetiology that is characterized by the infiltration of lymphocytes and macrophages into the cartilage; hyperplasia of the cartilage lining; enhanced angiogenesis and the destruction of cartilage bone. The pathogenesis of this disease is linked predominately to the formation of free radicals at the site of inflammation. Oxidative injury and the inflammatory state damage components of the extra-cellular matrix (ECM) either directly or indirectly by up-regulating mediators of matrix degradation. Reactive oxygen species (ROS) inhibit the synthesis of matrix components including proteoglycans by chondrocytes. In particular, ROS seem to inhibit type II collagen synthesis and the sulphation of newly synthesized glycosaminoglycans (GAGs). In addition, ROS can further damage the components of the ECM indirectly through the activation and up-regulation of matrix metalloproteinases (MMPs).

Although the nuclear factor kβ (NF-kβ) plays an essential and beneficial role in normal physiology, the up-regulation of this factor has been implicated in the pathogenesis of several diseases including inflammatory and rheumatic diseases. The p50 and p65 NF-kβ subunits are abundant in rheumatoid and osteoarthritic cartilage. Animal models of inflammatory arthritis also support the theory that NF-kβ plays a very active role in the development and progression of arthritis in vivo. NF-kβ signalling pathways mediate critical events in the inflammatory response via chondrocytes, leading to progressive ECM damage and cartilage destruction. Although oxidants are commonly considered to exert their effects via a direct toxic action on target cells, recent findings suggest their contributory role in gene induction. NF-kβ may, therefore, be activated by low levels of ROS and inhibited by antioxidants.

The dysregulation of apoptosis in the arthritic joint may contribute to the progression of the disease. Caspases are activated during the apoptotic process through a protease cascade, whereby the inactive pro-enzyme is cleaved to form subunits of the active heterotetrameric protease.
The degradative phase of apoptosis is characterized by membrane blebbing, mitochondrial dysfunction, cellular and cytoplasmic shrinkage and endonuclease activation and finally by cellular disintegration into small vesicles called apoptotic bodies. A great deal of evidence indicates that cell exposure to ROS induces apoptosis, which leads to cell death and results in tissue damage and impaired function. Apoptosis stimulated by different oxidant agents may be inhibited by a number of antioxidant compounds.

Most biological molecules have more than one function. In particular, many molecules have the ability to directly/indirectly scavenge free radicals and thus act as antioxidants in living organisms. One of these structures is the GAG chondroitin-4-sulphate (C4S), a biomolecule which has increasingly focused the interest of many research groups due to its antioxidant activity. The increase of C4S levels during oxidative stress seems to be a biological response that may protect cells from oxidation in synergy with the other antioxidant defense systems.

In the light of these findings, we investigated whether the administration of C4S, previously evaluated in collagen-induced arthritis (CIA) in rats, involves NF-kB and caspase modulation using a CIA model in mice.

Materials and methods

**ANIMALS**

Male mice DBA/16–7 weeks old with a mean weight of 25–30 g were used in our study. Mice, purchased from Harlan (Correzzana, Italy), were maintained under light-controlled conditions with a 12-h light/dark cycle. The animals were fed standard rodent chow and water ad libitum. The health status of the animal colony was monitored in accordance with Italian Veterinary Board guidelines. Mice were divided into the following groups: (1) Control (n = 21); (2) C4S (120 mg/kg) (n = 21); (3) CIA (n = 25); (4) CIA + C4S (30 mg/kg) (n = 21); (5) CIA + C4S (60 mg/kg) (n = 21); (6) CIA + C4S (120 mg/kg) (n = 21).

**MATERIALS**

C4S, at medium molecular weight (approximately 18,000 Da), from bovine trachea, approximately pure at 70% balanced with 30% C6S, was purchased from Fluka (division of Sigma). Bovine type II collagen and complete Freund's adjuvant were obtained from MD Biosciences (Zurich, Switzerland). Mouse MMP-13 polyclonal antibodies were obtained from Chemicon International, Inc. (Temecula, USA), caspase-3 and caspase-7 polyclonal antibodies and horseradish peroxidase-labelled goat anti-rabbit antibodies were obtained from Immunex Corporation (San Diego, USA). All other reagents were purchased from Fluka (division of Sigma–Alrich Dr, Milan, Italy).

**INDUCTION OF CIA AND ARTHRITIS EVALUATION**

Mice were injected intradermally at the base of the tail 100 μl of 2.0 mg/ml type II collagen emulsion in Freund's complete adjuvant containing 2.5 mg/ml heat-killed Mycobacterium tuberculosis H37Ra16. Mice were immunized a second time 21 days later. Evaluation of joint inflammation was carried out blindly by an independent observer with no knowledge of the treatment protocol. The severity of the arthritis in each limb was graded daily on a scale of 0–4 as follows: 0, no macroscopic signs of arthritis; 1, swelling of one joint of joints (i.e., wrist or ankle joints); 2, two groups of swollen joints; 3, three groups of swollen joints; 4, swelling of the entire limb. The maximum score for each rat was 16. Clinical severity was also assessed by quantifying changes in limb volume. Measurements were performed using a dial gauge caliper. At the end of the experimental period (day 45) mice were anaesthetized with ethyl ether and then were sacrificed to remove their hind limbs.

**C4S SECTION**

On day 20, coincident approximately with the onset of CIA, the animals were randomized to receive the treatments listed above in the Animals section. C4S was dissolved in saline solution (0.9% NaCl) and administered intraperitoneally using a volume of 1.0 ml/kg body weight, once a day up until day 45. The treatment of mice with C4S at 30, 60 and 120 mg/kg was chosen for the following reasons: (1) The chondroitin sulphate (CS) dosage in humans is, on average, 800 mg/day, that in a men weighing about 70 kg, corresponds to about 11.5 mg/kg. The treatment with this dosage is protracted for at least 10 months. We related this treatment with the short period in which our experiment was conducted, by administrating in 25 days the entire dosage that could be administered for 10 or 12 months. (2) The second reason was because we wanted to try a higher dosage than those we used in our previous experiments, in order to evaluate the ratio of increase in dosage/improvement of the disease. (3) The third reason was due to the high degree tolerance showed by C4S, allowing the possibility to assess its activity at high dosage without side effect or other pharmacological interferences.

NF-kB p50/65 TRANSCRIPTION FACTOR ASSAY

NF-kB p50/65 DNA binding activity in nuclear extracts of cartilage tissue samples was evaluated in order to measure the degree of NF-kB activation. Analysis was performed in line with the manufacturer's protocol for a commercial kit (NF-kB p50/65 transcription factor assay colorimetric, cat. no. SGT510, Chemicon International, USA). In brief, the hind limbs of the animals were removed at the end of the experimental period. Cartilage was separated from bone and muscular tissue, washed in ice-cold saline solution and then homogenized in ice-cold 10 mM Tris–HCl, pH 7.4, and blotted on absorbent paper. The homogenate was then resuspended in the appropriate extraction buffer and the nuclei were disrupted by a series of drawing and ejection actions. The subsequent analysis comprised a series of control steps accomplished in order to increase the specificity of the assay and to assure the nuclear extract whole cell extract tumour necrosis factor alpha (TNF-α) treated, transcription factor assay probe, NF-kB competitor oligonucleotide and NF-kB capture probe. After incubation with primary and secondary antibodies, colour development was observed following the addition of the substrate 1,2,3,4-tetramethylbenzidine (TMB/E). Finally, the absorbance of the samples was measured using a spectrophotometric microplate reader set at 450 nm. Values are expressed as relative optical density (OD)/mg protein.

IkBs ASSAY

IkBα loss was quantified in cartilage samples in order to confirm NF-kB activation. The test is based on solid phase sandwich enzyme-linked immunoassay (ELISA) assay. The cytosolic fraction, obtained during the nuclei extraction procedure for NF-kB assay, was assayed for IkBα evaluation. The assay was carried out using a commercial kit (IkBS, Total Human Bio-Assay ELISA Kit, cat. no. 12500-05T, USBiological, USA). Values are expressed as relative OD/mg protein.

**RNA ISOLATION, CDNA SYNTHESIS AND REAL TIME QUANTITATIVE POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION**

Total RNA was isolated from cartilage tissue for reverse-PCR real time analysis of MMP-13, caspase-3 and caspase-7 (RealTime PCR system, Mod. 7500, Applied Biosystems, USA) using an Omnisol Reagent Kit (Eurclone, West York, UK). The first strand of cDNA was synthesized from 1.0 μg total RNA using a high capacity cDNA Archive kit (Applied Biosystems, USA). 3-actin mRNA was used as an endogenous control to allow the relative quantification of MMP-13 and caspase mRNAs. PCR real time was performed by means of ready-to-use assays (Assays on demand, Applied Biosystems) on both targets and endogenous controls. The amounts of specific mRNA in samples were calculated from the standard curve, and normalized with the β-actin mRNA. After normalization, the mean value of normal cartilage cell target levels became the calibrator (one/sample) and the results are expressed as the n-fold difference relative to normal controls (relative expression levels).

**WESTERN BLOT ASSAY OF MMP-13, CASPASE-3 AND CASPASE-7 PROTEINS**

For SDS-PAGE and Western blotting, the cartilage cells were washed twice with cold phosphate buffer, then homogenized in SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromophenol blue). Aliquots of whole cell protein extract (10–25 μl) were separated on a mini gel (10%). The proteins were blotted onto polyvinylidene difluoride membranes (Amersham Biosciences) using a semi-dry apparatus (Bio-Rad). The membranes were then incubated with the specific diluted (1:1000) primary antibody in 5%...
bovine serum albumin, 1% PBS, and 0.1% Tween 20 at 4°C overnight in a roller bottle. After being washed in three stages in wash buffer (1× PBS, 0.1% Tween 20), the blots were incubated with the diluted (1:2500) secondary polyclonal antibody (goat anti-rabbit conjugated with peroxidase), in tris buffered saline (TBS)/Tween-20 buffer, containing 5% non-fat-dried milk. After 45 min of gentle shaking, the blots were washed five times in wash buffer, and the proteins, after coloration with 3,3-diaminobenzidine (DAB) liquid substrate (Sigma–Aldrich, Milan, Italy) were made visible using an UV/visible transilluminator (EuroClone, Milan, Italy) and Kodak BioMax MR film. A densitometric analysis was also run in order to quantify each band.

LIPID PEROXIDATION ESTIMATION

Determination of 8-isoprostane (8-IPE) in the articular cartilage was carried out to evaluate the extent of oxidation of cartilage phospholipids by oxygen radicals. The analysis was performed using an EIA commercial kit (8-Isoprostane EIA Kit, cat. no. 516351; Cayman Chemical Company, Ann Arbor, USA). Values are expressed as relative OD/mg of protein.

POLYMORPHONUCLEAR (PMN) ACTIVATION

Elastase (ELA) activity was assayed as an index of neutrophil accumulation and activation in cartilage tissue. The analysis was performed using a fluorometric commercial kit (Elastase Assay Kit, EnzChek, cat. no. E12056, Invitrogen, Eugene, USA). Values are expressed as fluorescent arbitrary units (FAUs) at Ex 480 nm and Em 520 nm/mg protein.

CATALASE (CAT) ANALYSIS

For CAT activity, cartilage tissue was homogenized in a 1:10 (w/v) ratio with an Ultra-Turrax homogenizer, maintained at 4°C, in ice-cold 50 mM potassium phosphate buffer pH 7.4. The homogenate was then centrifuged at 5000 g for 10 min, and the supernatant (100 μl) was added to a tube containing 10 μl of protease inhibitor cocktail and 0.1 mM EDTA sodium salt. CAT activity was then assayed spectrophotometrically using a commercial kit (Catalase-520, cat. no. 21042, OxisResearch, Portland, OR, USA). Values are expressed as units/min/mg protein.

REDUCED GLUTATHIONE (GSH) ASSESSMENT

For GSH evaluation, cartilage tissue samples were homogenized in a 1:10 (w/v) ratio using an Ultra-Turrax homogenizer, maintained at 4°C, in a solution containing 5% metaphosphoric acid (MPA) acid and 5 mM ethylenediamine tetraacetic acid (EDTA). Each sample was then centrifuged at 6000 g for 10 min at 4°C. Biochemical analysis was performed using a specific colorimetric assay (Bioxyn GSH-400 Assay Kit, cat. no. 21011, OxisResearch, Portland, OR, USA). The amount of cartilage GSH was expressed as nmol/mg protein.

HISTOLOGY

At the end of the experimental phase, knee joints were removed from hind limbs of the animals and fixed in 10% buffered formalin. Knee joints were decalcified in 5% formic acid, processed for paraffin embedding, sectioned at 5 μm thickness and subsequently stained with Safranin O for examination under a light microscope (Optech Instrument, Munchen, Germany) connected up to a digital camera (Coolpix 4500, Nikon, Japan).

PROTEIN ANALYSIS

The amount of protein was determined using the Bio-Rad protein assay system (Bio-Rad Laboratory, Richmond, CA, USA) with bovine serum albumin as a standard in accordance with the published method23.

STATISTICAL ANALYSIS

Data are expressed as means ± S.D. of no less than seven experiments for each test. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. The statistical significance of differences was set at P < 0.05.

STATEMENT OF ANIMAL CARE

The studies reported in this manuscript have been performed out in accordance with the Helsinki declaration and the guidelines for the Care and Use of Laboratory Animals.

Results

EFFECTS OF C4S ON CLINICAL SIGNS OF CIA

Some days after the second immunization, animals began to show evidence of clinical inflammation in one or more hind limbs. The first manifestation of disease was erythema of one or more ankle joints, followed by the metatarsal and interphalangeal joints. Figure 1(A) reports the incidence of CIA over the 45-day study period. The initial signs of arthritis in all groups were evident at day 25. In the CIA group the percentage of mice developing arthritis was 98.8 at day 25 and the same percentage was maintained until the end of the experiment. Treatment with C4S significantly attenuated the development of CIA: 76% with the dose of 30 mg/kg; 54% with the dose of 60 mg/kg and, at 40% with the dose of 120 mg/kg [Fig. 1(A)].

At the end of the experiment, the final state of disease, as assessed by the arthritis evaluation scale, is shown in Fig. 1(B). By day 25 all animals showed evidence of the disease, predominantly in the hind paws. The disease was always progressive with joint recruitment following the same pattern: tarsal, metatarsophalangeal followed by the interphalangeal. The interphalangeal joints were never involved alone, and inflammation in these joints was invariably associated with inflammation in the tarsal joint. The arthritis score in CIA+ vehicle group was progressive from day 25 and reached the score of about 12 in the last 4 days.

![Fig. 1. Effect of C4S treatment on the development (A) and progression (B) of arthritis at the end of experiment. Evaluation was performed at the end of the experiment (day 60). Values are the mean ± S.D. of seven experiments. *P < 0.001 vs Control; **P < 0.005 and ***P < 0.001 vs CIA.](image-url)
The same changes were observed in the hind paw diameter of CIA animals. Administration of C4S significantly attenuated all clinical signs of arthritis.

**NF-kB DNA BINDING ACTIVITY**

Figure 2(A) shows the changes in NF-kB p50/65 heterodimers in the nuclear extract of cartilage cells. NF-kB DNA binding was present at very low levels in CIA-untreated cartilage cells, and the p50 subunit was more represented than the p65 subunit. In contrast, NF-kB was markedly increased in mice with CIA and also the ratio p50/p65 was inverted.

The chronic treatment of mice with C4S resulted in a significant inhibition of NF-kB DNA binding. The effect was positive at all the doses used [Fig. 2(A)].

**LOSS OF IkBα PROTEIN IN THE CYTOPLASM OF CARTILAGE CELLS**

NF-kB activation was also indirectly investigated by studying its inhibitory protein IkBα in the cytoplasm of cartilage cells [Fig. 2(B)]. Physiological levels of IkBα protein were assayed in CIA-untreated mice, while arthritis produced a significant reduction in IkBα protein in the cytoplasm of cartilage cells.

![Figure 2](image-url)
The treatment of mice with C4S significantly blunted the loss of IkBα protein in the cytoplasm.

MMP-13 mRNA EXPRESSION AND PROTEIN ACTIVITY

MMP-13 was evaluated because of its active role in the cartilage destruction [Fig. 3(A–C)]. Quantification of gene expression [Fig. 3(A)] showed that the mRNA of this protein was not stimulated in CIA-untreated mice. However, in the animals subjected to CIA MMP-13 expression was significantly up-regulated. The chronic administration of C4S was able to reduce this increment in mRNA. The increase in MMP-13 expression in arthritic mice correlated well with the increment in protein synthesis. This correlation also held for mice treated with C4S. In fact, the reduction in MMP-13 expression corresponded with a similar diminution in protein formation [Fig. 3(B and C)].

CASPASE-3 AND CASPASE-7 mRNA EXPRESSION AND PROTEIN LEVELS

The apoptotic activators caspase-3 and caspase-7 were evaluated by measuring mRNA expression and protein activity in order to estimate apoptosis in cartilage tissue. Caspase-3 [Fig. 4(A)] and caspase-7 [Fig. 5(A)] mRNA evaluation and protein expression [Figs. 4(B and C) and 5(B and C)] in animals with CIA showed a marked increase in the expression of the two apoptotic proteases. The treatment of mice with C4S was able to reduce caspase-3 and caspase-7 mRNA expression and new protein generation.
Once again, caspase-3 and caspase-7 mRNA levels correlated well with the protein concentrations obtained by Western blot analysis.

8-IPE EVALUATION

Determination of 8-IPE in articular cartilage was carried out in order to estimate the lipid peroxidation in the cartilage tissue (Table I). Low levels of 8-IPE were measured in CIA-untreated mice whereas, a significant increment in this marker was found in the joint cartilage of mice with CIA. Chronic treatment with C4S was able to reduced 8-IPE levels in the cartilage tissue.

ELA ACTIVITY

Neutrophil activation contributed to the progression and the diffusion of inflammation in the cartilage tissue (Table I). Very low ELA activity was measured in CIA-untreated mice. In contrast, elevated ELA activity levels were assayed in the CIA group. The administration of C4S was able to reduce PMN accumulation in the cartilage as demonstrated by the decreased ELA activity levels.

ENDOGENOUS ANTIOXIDANTS

The CAT activity and total GSH levels (Table II) were assayed in order to evaluate the endogenous antioxidant.
Fig. 5. Effect of C4S treatment on the articular cartilage caspase-7 mRNA expression (A) and related protein production (B, C) in joints of mice with CIA. Values are the mean ± S.D. of seven experiments and are expressed as the n-fold increase with respect to the Control (A) and as both densitometric analysis (C) and Western blot analysis (B) for the caspase-7 protein levels. *P < 0.05, **P < 0.005 and ***P < 0.001 vs CIA.

Table I
Effect of C4S treatment on the articular cartilage 8-IPE content and ELA levels in joints cartilage of mice with CIA

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>8-IPE</th>
<th>ELA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.3 ± 0.2</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>C4S (120 mg/kg)</td>
<td>1.5 ± 0.3</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>CIA</td>
<td>37.8 ± 7.4°C</td>
<td>2.78 ± 0.37°C</td>
</tr>
<tr>
<td>CIA + C4S (30 mg/kg)</td>
<td>24.2 ± 6.2*</td>
<td>2.11 ± 0.32*</td>
</tr>
<tr>
<td>CIA + C4S (60 mg/kg)</td>
<td>16.5 ± 4.7**</td>
<td>1.81 ± 0.30**</td>
</tr>
<tr>
<td>CIA + C4S (120 mg/kg)</td>
<td>9.8 ± 4.1**</td>
<td>1.73 ± 0.34**</td>
</tr>
</tbody>
</table>

Values are the mean ± S.D. of seven experiments and are expressed as pg/mg protein for 8-IPE and as FAUs at λ Ex 480 nm and λ Em 520 nm/mg protein for ELA. *P < 0.005 vs CIA.

Table II
Effect of C4S treatment on the articular cartilage CAT activity and reduced GSH levels in joints of mice with CIA

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>CAT</th>
<th>Reduced GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>120.3 ± 15.2</td>
<td>8.8 ± 1.8</td>
</tr>
<tr>
<td>C4S (120 mg/kg)</td>
<td>123.1 ± 17.4</td>
<td>8.5 ± 1.8</td>
</tr>
<tr>
<td>CIA</td>
<td>50.6 ± 8.3°C</td>
<td>3.2 ± 0.8°C</td>
</tr>
<tr>
<td>CIA + C4S (30 mg/kg)</td>
<td>71.2 ± 9.1*</td>
<td>5.8 ± 1.4*</td>
</tr>
<tr>
<td>CIA + C4S (60 mg/kg)</td>
<td>83.7 ± 9.5**</td>
<td>6.4 ± 1.5**</td>
</tr>
<tr>
<td>CIA + C4S (120 mg/kg)</td>
<td>94.8 ± 10.3**</td>
<td>7.2 ± 1.6**</td>
</tr>
</tbody>
</table>

Values are the mean ± S.D. of seven experiments and are expressed as units/min/mg protein for CAT and as nmol/mg protein for reduced GSH. *P < 0.001 vs Control; *P < 0.005 and **P < 0.001 vs CIA.
defences after CIA. A significant reduction in both these antioxidants was observed in the cartilage tissue obtained from mice subjected to CIA. Once again, the treatment with C4S significantly restored CAT and GSH activities.

**HISTOLOGICAL ANALYSIS**

Figure 6 reports a representative joint histopathology of all the experimental groups considered. Panels A and B...
show a knee joint section from CIA-untreated mice. The fully stained cartilage layers indicate no loss of proteoglycans or erosion. Panel C presents a joint section from a CIA-treated mouse. This clearly shows the severe knee joint inflammation and complete loss of Safranin O staining of the cartilage layers (indicated by arrows). In panels D, E, and F a representative selection of knee joint sections joints from mice receiving CIA and C4S treatment shows that a gradual reduction in proteoglycan depletion and cartilage erosion occurred (Fig. 6).

Discussion

Arthritis is a disease affecting all the entire system, although the skeletal apparatus is the principal target such as the joint, including the cartilage, the subchondral bone, and the periarticular tissues. The metabolic and structural changes in the articular cartilage are thought to play a leading role in the initiation and the progression of the disease process. The pathogenesis of arthritis is multifactorial and several factors are involved. A number of authors have focused their attention on ROS as potent mediators of cartilage erosion. Cartilage degradation, which is likely to be driven by biomechanical factors, results in an imbalance between catabolic and anabolic activities. MMPs and ROS are the two main agents of matrix component degradation. They have been found to be overproduced in arthritic cartilage.

Modulation of NF-kB activation could be an important strategy to reduce cellular injury. There is in fact increasing evidence to suggest that NF-kB is an important mediator in the pathophysiology of diseases characterized by elevated levels of cytokines and ROS such as sepsis and inflammation. ROS are also involved in the regulation of interleukin-1 (IL-1) effects mediated by NF-kB. NF-kB up-regulates genes (including MMPs) that are involved in cartilage degradation. This degradation involves the enhanced cleavage of type II collagen by collagenases, particularly collagenase-3 (MMP-13). This is associated with increased type II collagen denaturation. Several antioxidant compounds have been found to be able to reduce NF-kB activation with a consequent reduction in cell damage.

Oxidative stress is able to produce many intracellular events including apoptosis. There are two major pathways via which apoptosis is induced; one involves death receptors and is exemplified by Fas-mediated caspase-8 activation, and the other is the stress- or mitochondria-mediated caspase-9 activation pathway. Both pathways converge on caspase-3 and caspase-7 activation, resulting in nuclear degradation and cellular morphological change. Oxidative stress induces cytochrome c release from mitochondria and activation of caspases, p53, and kinases. Apoptosis contributes heavily to cell death in rheumatoid arthritis (RA). It has been reported that molecules capable of scavenging ROS may reduce apoptosis activation and chondrocyte disruption.

It has been suggested that CSs inhibit ROS production. CSs are the most abundant GAGs present in the human organism, and they can be localized anywhere in connective tissues. Moreover, CSs are the most common components of circulating GAGs. They are also constituents of normal urine, and are present in leukocytes and platelets. These molecules may be distinguishable as a function of their degree of sulphation at the C-4 or C-6 positions of galactosamine. C4S has exhibited the greatest antioxidant effect of CS.

In the present study the treatment of arthritic mice with different doses of C4S was able to prevent cell damage induced by CIA treatment as shown in our previous experiments on different type of animal. In addition to the antioxidant effect of this GAG, the focus of this study was the C4S activity on NF-kB and executioner caspases modulation. We hypothesized that the inhibition of NF-kB DNA binding to the nucleus is probably the consequence of C4S reduced ROS production in articular cartilage. NF-kB activation requires sequential phosphorylation, and degradation of IkBz, which disappears from the cytoplasm in the end. Since ROS are able to activate this pathway, then C4S is also supposed to prevent the loss of the inhibitory protein from the cytoplasm by preventing the oxidative burst. Although several papers have reported that CS may also directly inhibit NF-kB activation and apoptosis, we suggest that the same CS mechanism hypothesized for NF-kB DNA binding exerted by ROS may be extended to apoptosis activation. In fact, the reduction of oxidative stress was able to limit the caspase activation pathway. C4S thereby reduced damage not only by reducing ROS generation, but also by inhibiting NF-kB and apoptosis activation that contributes greatly to exacerbate pathological conditions in diseases. It has been hypothesized that C4S exerted its antioxidant effect by chelating transition metal ions, since Haber-Weiss and Fenton reactions are primed by transition metal ions such as Fe3+. The effect of the chelation of this metal ion reduces oxidative stress by eliminating the products of this reaction, and it is reasonable to hypothesize that acting by blocking this reaction is more efficient than scavenging the reactive activity of the products formed. In this way, C4S activity would be more efficient than a conventional antioxidant, because the inhibition of the oxidative burst does not prime NF-kB and apoptosis. If this were so, the antioxidant effect of C4S would be independent from NF-kB and caspase activation. Since the treatment of mice with C4S was able to protect chondrocytes from oxidative injury and also to inhibit NF-kB DNA binding and apoptosis with two separate mechanisms, this study suggests that the antioxidant effect of C4S is due to the blocking of Haber-Weiss and Fenton’s reactions by metal ion chelation. Nevertheless, cannot be also excluded that C4S may exert a direct effect on NF-kB and apoptosis inhibition. Additional future investigations are needed to confirm this further hypothesis. Therefore, since in arthritis injury several different pathways are involved, the antioxidant hypothesis is one potential mechanism worthy of interest.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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