Glycosaminoglycans Modulate Inflammation and Apoptosis in LPS–Treated Chondrocytes

Giuseppe M. Campo,* Angela Avenoso, Salvatore Campo, Angela D'Ascola, Paola Traina, Dario Samà and Alberto Calatroni

Department of Biochemical, Physiological and Nutritional Sciences, Section of Medical Chemistry, School of Medicine, University of Messina, Policlinico Universitario, 98125 Messina, Italy

ABSTRACT

Previous studies reported that hyaluronic acid (HA), chondroitin sulphate (CS) and heparan sulphate (HS) were able to reduce the inflammatory process in a variety of cell types after lypopolysaccharide (LPS) stimulation. The aim of this study was to investigate the anti-inflammatory effect of glycosaminoglycans (GAGs) in mouse articular chondrocytes stimulated with LPS. Chondrocyte treatment with LPS (50 μg/ml) generated high levels of TNF-α, IL-1β, IL-6, IFN-γ, MMP-1, MMP-13, iNOS gene expression and their related proteins, increased NO concentrations (evaluated in terms of nitrites formation), NF-κB activation and IkBα degradation as well as apoptosis evaluated by the increase in caspase-3 expression and the amount of its related protein. The treatment of chondrocytes using two different doses (0.5 and 1.0 mg/ml) of HA, chondroitin-4-sulphate (C4S), chondroitin-6-sulphate (C6S), HS, keratan sulphate (KS) and dermatan sulphate (DS) produced a number of effects. HA exerted a very small anti-inflammatory and anti-apoptotic effect while it significantly reduced NO levels, although the effect on iNOS expression and activity was extremely slight. C4S and C6S reduced inflammation mediators and the apoptotic process. C6S failed to decrease NO production, although iNOS expression and activity were significantly reduced. HS, like C4S, was able to reduce all the effects stimulated by LPS treatment. KS and DS produced no reduction in any of the parameters considered. These results give further support to the hypothesis that GAGs actively participate in the regulation of inflammatory and apoptotic processes. J. Cell. Biochem. 106: 83–92, 2009. © 2008 Wiley-Liss, Inc.

KEY WORDS: GLYCOSAMINOGLYCANS; LPS; CHONDROCYTES; INTERLEUKINS; CASPASES

Inflammation is a multiple cascade process mediated by activated inflammatory or immune cells. During this process a number of immunopathological changes occur: including the overproduction of pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interferon gamma (IFN-γ), nitric oxide (NO) and other detrimental mediators, including caspases that in turn activate the apoptotic process [Wesche-Soldato et al., 2007; Herrington and Hall, 2008]. The over-production of TNF-α stimulates the generation of the other pro-oxidant mediators that in turn directly produce cell injury. Among these, free radicals, such as NO, seem to play a central role in cell damage [Weinberg et al., 2007].

Lypopolysaccharide (LPS) is the principal component of the outer membrane of Gram-negative bacteria. The cell response to LPS includes the expression of a variety of inflammatory cytokines and cytotoxic mediators that can lead to sepsis, septic shock, or systemic inflammatory response syndrome [Guha and Mackman, 2001]. These processes may also involve caspase activation that in turn lead to apoptosis and cell death. It is also known that all these events are modulated mainly by nuclear transcription factor NF-κB activation which is greatly involved in the regulation of inflammation [Brasier, 2006].

Glycosaminoglycans (GAGs) are long, linear and heterogeneous polysaccharides that play a role in many biological functions, including growth control, signal transduction, cell adhesion, haemostasis and lipid metabolism [Tumova et al., 2000; Volpi, 2006]. GAGs play a critical role in assembling protein–protein complexes such as growth factor receptors or enzyme inhibitors on the cell surface and in the extracellular matrix that are directly involved in initiating cell signalling events or inhibiting biochem-

ical pathways [Raman et al., 2005]. GAGs also participate in pathological processes, such as inflammation [Gozzo et al., 2002], microbial pathogenesis [Chiu et al., 2007] and cancer [Monzavi-Karbassi et al., 2007]. Nevertheless, despite these vital functions, our current level of knowledge seems to be extremely limited since GAGs seem to perform several other important functions that are so far unknown.

Recently, several studies have shown the antioxidant properties of GAGs, mainly for hyaluronic acid (HA) and chondroitin-4-sulphate (C4S) using both in vitro and in vivo experimental models [Albertini et al., 1999; Arai et al., 1999; Balogh et al., 2003; Campo et al., 2003a,b]. This antioxidant activity is due to their capacity to chelate transition metals, like Cu^{++} or Fe^{++}, that are in turn responsible for the initiation of Haber–Weiss and Fenton's reaction [Albertini et al., 1999; Balogh et al., 2003; Campo et al., 2004]. It was also reported that HA and C4S were able to reduce cell injury by inhibiting NF-κB and caspase activation during oxidative stress [Campo et al., 2008]. The inhibitory activity on NF-κB and apoptosis activation was probably exerted by reducing oxygen species production, since free radicals may directly activate NF-κB [Gloire et al., 2006]. Although this antioxidant mechanism is well supported by several investigations, possible additional direct activity of HA and C4S on NF-κB inhibition cannot be excluded. In fact, previous investigations reported that the GAGs: HA, chondroitin sulphates (CS) and heparan sulphate (HS) were able to reduce the inflammatory process in a variety of cell types after LPS stimulation [Asakura et al., 2004; Holzmann et al., 2006; Yasuda, 2007].

In light of these findings, the aim of the present study was to investigate the anti-inflammatory activity of GAGs using an experimental model of LPS-induced toxicity in cultured mouse articular chondrocytes.

MATERIALS AND METHODS

MATERIALS

LPS from *Salmonella enteritidis*, HA medium molecular weight sodium salt from *Streptococcus equi*, C4S sodium salt from bovine trachea, chondroitin-6-sulphate (C6S) sodium salt from shark cartilage, HS sodium salt from bovine kidney and dermatan sulphate (DS) sodium salt from porcine intestinal mucosa were all obtained from Sigma–Aldrich S.r.l. (Milan, Italy). Keratan sulphate from bovine cornea was purchased from Seikagaku Corporation (Tokyo, Japan). Mouse MMP-1 and MMP-13 polyclonal antibodies were purchased from Chemicon International, Inc. (Temecula), mouse TNF-α, IL-1β, IL-6, inducible nitric oxide synthase (iNOS), caspase-3 polyclonal antibodies and horseradish peroxidase-labeled goat anti-rabbit antibodies were obtained from GenWay Biotech Inc. (San Diego). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, trypsin–ethylenediaminetetraacetic acid (EDTA) solution and phosphate buffered saline (PBS) were obtained from GibcoBRL (Grand Island, NY). All cell culture plastics were obtained from Falcon (Oxnard, CA). Sulphanilamide, naphthyl-ethylenediamine dihydrochloride, phosphoric acid, sodium nitrite, sucrose, EDTA, potassium phosphate, biotin-11-dUTP, butylated hydroxytoluene (BHT), diamino-benzidine (DAB), dichloromethane (DCM), deoxynucleotidyl transferase (TdT), trypan blue, RNase, proteinase K, protease inhibitor cocktail, sodium dodecyl sulphate (SDS) and all other general laboratory chemicals were obtained from Sigma–Aldrich S.r.l.

Cell cultures. Normal knee mouse chondrocytes (DPK-CACC-M, strain: C57BL/6J, Dominion Pharmakine, Bizkaia, Spain) were cultured in 75 cm^2 plastic flasks containing 15 ml of DMEM supplemented with 10% FBS, L-glutamine (2.0 mM) and penicillin/streptomycin (100 U/ml, 100 μg/ml), and incubated at 37°C in humidified air with 5% CO_2.

LPS stimulation and GAG treatment. Chondrocytes were cultured into six-well culture plates at a density of 1.3×10^5 cells/well. Twelve hours after plating (time 0) the culture medium was replaced with 2.0 ml of fresh medium containing LPS at a concentration of 50 μg/ml. Four hours later, HA, C4S, C6S, HS, keratan sulphate (KS) and DS were added separately to each of the wells at concentrations of 0.5 and 1.0 mg/ml. Finally, 24 h later, cells and medium were subjected to morphological and biochemical evaluation.

Cell viability assay. The viable chondrocytes were determined under a photozoom invert microscope (Optech GmbH, Munchen, Germany) connected to a digital camera (mod. Coolpix 4500, Tokyo, Japan). The number of viable chondrocytes was then quantified by trypan blue dye exclusion test from several randomly chosen areas of each well.

RNA isolation, cDNA synthesis and real-time quantitative PCR amplification. Total RNA was isolated from chondrocytes for reverse transcription-PCR real time analysis of TNF-α, IL-1β, IL-6, IFN-γ, iNOS, MMP-1, MMP-13 and caspase-3 (RealTime PCR system, Mod. 7500, Applied Biosystems) using an Omnizol Reagent Kit (Euroclone, 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). β-actin mRNA was used as an endogenous control to allow the relative quantification of TNF-α, IL-1β, IL-6, IFN-γ, iNOS, MMP-1, MMP-13 and caspase-3 mRNAs [Bustin, 2000]. PCR real-time was performed by means of ready-to-use assays (Assays on demand, Applied Biosystems) on both targets and endogenous controls. The amplified PCR products were quantified by measuring the calculated cycle thresholds (C_T) of TNF-α, IL-1β, IL-6, IFN-γ, iNOS, MMP-1, MMP-13, caspase-3 and β-actin mRNA. The C_T values were plotted against the log input RNA concentration in serially diluted total RNA of chondrocyte samples and used to generate standard curves for all mRNAs analysed. 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 per sample) and the results are expressed as the n-fold difference relative to normal controls (relative expression levels).

Western blot assay of TNF-α, IL-1β, IL-6, IFN-γ, iNOS, MMP-1, MMP-13 and caspase-3 proteins. For SDS–PAGE and Western blotting, chondrocytes were washed twice in ice-cold PBS and

subsequently dissolved 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). β-actin protein was used as an endogenous control to allow the normalization of TNF-α, IL-1β, IL-6, IFN-γ, iNOS, MMP-1, MMP-13 and caspase-3 proteins. Aliquots of whole cell protein extract (10–25 μl/well) 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 (1:1,000) 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 (1:2,500) secondary polyclonal antibody (Goat anti-rabbit conjugated with peroxidase), in 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 DAB liquid substrate (Sigma–Aldrich) were made visible using an UV/visible transilluminator (EuroClone, Milan, Italy and Kodak BioMax MR films). An automated densitometric analysis was also run (Kodak Image Station 2000R) in order to quantify each band.

NF-kB p50/65 transcription factor assay. NF-kB p50/65 DNA binding activity in nuclear extracts of chondrocytes was evaluated in order to measure the degree of NF-κB activation. Analysis was performed in line with the manufacturer's protocol for a commercial kit (NF-kB p50/65 Transcription Factor Assay Colorimetric, cat. n°SGT510, Chemicon International). In brief, the. cytosolic and nuclear extraction was performed by lysing the cell membrane with an apposite hypotonic lysis buffer containing protease inhibitor cocktail and tributylphosphine (TBP) as reducing agent. The lysate was then incubated in the buffer on ice and centrifuged at 250g. A series of drawing and ejecting actions were performed using a syringe with a small gauge needle. This step was carried out five times. After centrifugation at 8,000g the supernatant containing the cytosolic portion of cell lysate was recovered and stored at −70°C for subsequent analysis. The pellet containing the nuclear portion was then re-suspended in the apposite extraction buffer and the nuclei were disrupted by a series of drawing and ejecting actions. After gentle stirring for 40 min, the nuclei suspension was centrifuged at 16,000g. The supernatant fraction was the nuclear extract. After the determination of protein concentration and adjustment to a final concentration of approximately 4.0 mg/ml, this extract was stored in aliquots at −80°C for the subsequent NF-κB assay. The analysis comprised a series of control steps accomplished by adding the following to the nuclear extract: HeLa whole cell extract (TNF-α treated), transcription factor assay probe, NF-κB competitor oligonucleotide, NF-κB capture probe and enhanced transcription factor assay buffer. This procedure was carried out in order to obtain the transcription factor assay as: normal, positive control, specific competitor control and negative control. After incubation with primary and secondary antibodies, colour development was observed following the addition of the substrate TMB/E. Finally, the absorbance of the samples was measured using a spectrophotometric microplate reader set at λ

450 nm. Values are expressed as relative optical density (OD) per mg protein.

IkBα assay. IkBα loss was quantified in chondrocytes in order to confirm NF-κB activation. The test is based on solid phase sandwich ELISA assay. The cytosolic fraction, which was obtained during the nuclei extraction procedure for NF-κB assay, was used for IkB-α evaluation. The assay was carried out using a commercial kit (IkBα, Total Human BioAssay ELISA Kit, cat. n°12500-05T, USBiological). Briefly, 100 μl of solution of standards, samples and controls were added to each well of the coated microplate. After 2 h of incubation at room temperature, the microplate was decanted and the liquid discarded. Wells were washed four times. Subsequently, 100 μl of anti-IkB-α antibodies were added to each well. After 1 h incubation at room temperature, the liquid was again removed from the wells and these were washed four times, 100 μl of anti-rabbit IgG-HRP was added. After further incubation for 30 min and having washed the wells four times, 100 μl of stabilized chromogen was added. Absorbance was measured using a spectrophotometric microplate reader set at λ 450 nm. Values are expressed as relative optical density (OD)/mg protein.

TUNEL assay. TUNEL assay was performed with the in situ cell apoptosis detection kit (Roche Diagnostics, Basel, Switzerland) following its manufacturer's instructions. Apoptotic chondrocytes were visualized using a fluorescence microscopy (Carl Zeiss GmbH, Oberkochen, Germany). In brief, at the end of the experiments chondrocytes were replanted on cover slips and fixed with 4% paraformaldehyde for 30 min and adhered to slides with balsam. Non-specific chromogen reaction, induced by endogenous peroxidase, was inhibited with 3% H_2O_2 for 10 min at room temperature. Terminal deoxynucleotidyl transferase (TdT) and biotin-11-dUTP

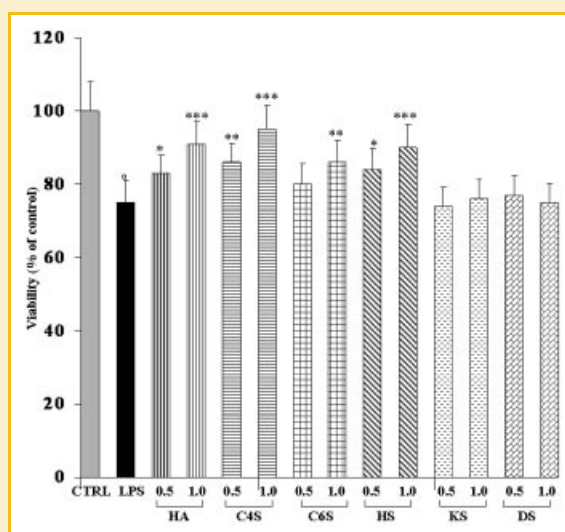Fig. 1. Effect of GAG treatment on chondrocyte survival. GAGs were added to chondrocytes 4 h after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as percentages with respect to control cells. °$P < 0.001$ versus control; *$P < 0.01$, **$P < 0.005$ and ***$P < 0.001$ versus LPS.

reactions were performed for 1 h at 37°C in a humidified box, and blocking reagent was applied for 30 min at room temperature, followed by avidin-HRP for 1 h at 37°C in a humidified box. For biochemical controls, positive control slides were treated with DNase and negative control slides were treated with PBS instead of TdT. DNA fragments were stained using DAB as substrate for peroxidase, and hematoxylin was used as counter stain. Apoptotic index was calculated as a ratio of the number of apoptotic cells to the total number of chondrocytes in each slide.

NO release assay. At the end of the experiments, the culture media were removed and assayed for nitrite production (a stable metabolite byproduct of NO generation) using Greiss reagent (1%

sulphanilamide/0.1% naphthyl-ethylenediamine dihydrochloride/2.5% phosphoric acid). In brief, 0.1 ml medium or sodium nitrite standards were transferred to a 96-well plate, followed by the addition of 0.1 ml of Greiss reagent. The absorbance values were read at λ 540 nm on an automated microtiter plate reader (DAS srl, Rome, Italy). NO levels were calculated with reference to the standard curve of sodium nitrite generated by known concentrations. NO levels are expressed as nmol/mg protein.

Protein determination. The amount of protein was determined using the Bio-Rad protein assay system (Bio-Rad Lab., Richmond, CA) with bovine serum albumin as a standard in accordance with the published method [Bradford, 1976].
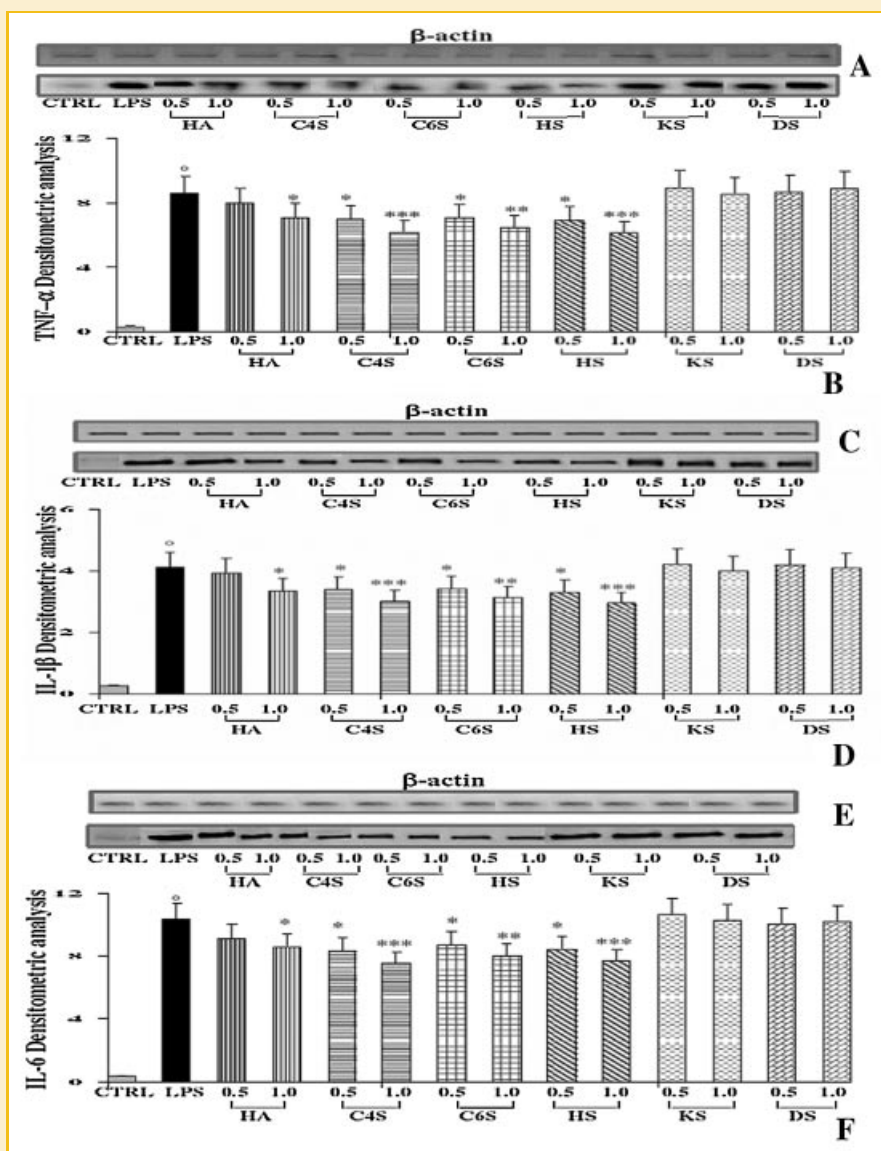

Fig. 2. Effect of GAG treatment on chondrocyte TNF-α, IL-1β and IL-6 protein production levels. GAGs were added to chondrocytes 4 h after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as both Western Blot analysis (panels A, C, E) and densitometric analysis (arbitrary units) (panels B, D, F). °$P < 0.001$ versus control; *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ and ****$P < 0.001$ versus LPS.

Statistical analysis. Data are expressed as means ± SD of no less than seven experiments for each test. All assays were repeated three times to ensure reproducibility. 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 $P < 0.05$.

RESULTS

EFFECTS OF GAGS ON CELL VIABILITY

The exposure of chondrocytes to LPS produced significant mortality and growth inhibition, as shown in Figure 1. Cell mortality rate was around 25%. The treatment with GAGs produced the following

effects: HA, C4S and HS provided significant protection to cells at both the doses used, while C6S decreased mortality only when using the highest dose, and KS and DS did not exert any protective effect (Fig. 1).

TNF-α, IL-1β, IL-6, IFN-γ, INOS, MMP-1, MMP-13 AND CASPASE-3 WESTERN BLOT ANALYSIS AND mRNA EXPRESSION

TNF-α, IL-1β, IL-6 (Fig. 2), IFN-γ, iNOS, MMP-1 (Fig. 3), MMP-13 and caspase-3 (Fig. 4) Western blot analysis (panels A, C, E of each figure) with densitometric evaluation (panels B, D, F of each figure) showed a marked increase in the protein synthesis of all inflammatory cytokines, the iNOS, MMPs and the apoptotic
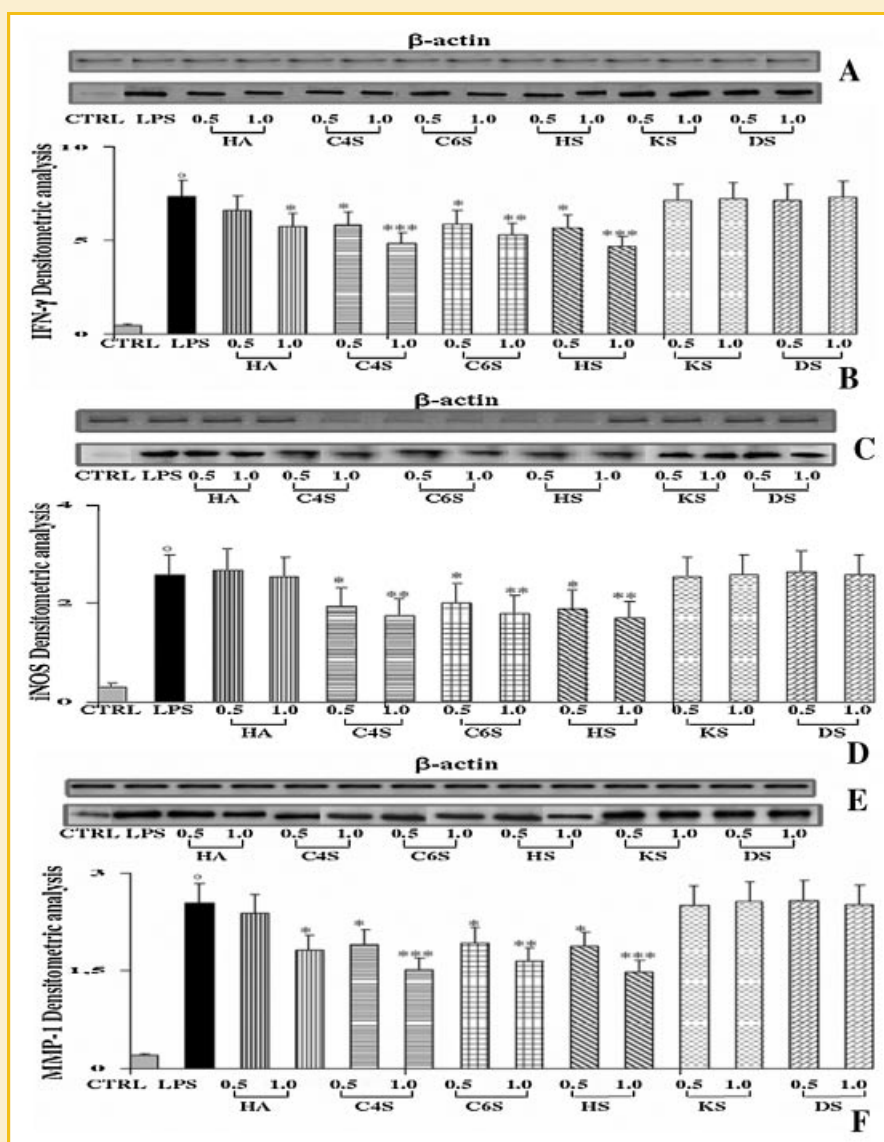

Fig. 3. Effect of GAG treatment on chondrocyte IFN-γ, iNOS and MMP-1 protein production levels. GAGs were added to chondrocytes 4 h after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as both Western Blot analysis (panels A, C, E) and densitometric analysis (arbitrary units) (panels B, D, F). $°P < 0.001$ versus control; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ and $^{****}P < 0.001$ versus LPS.
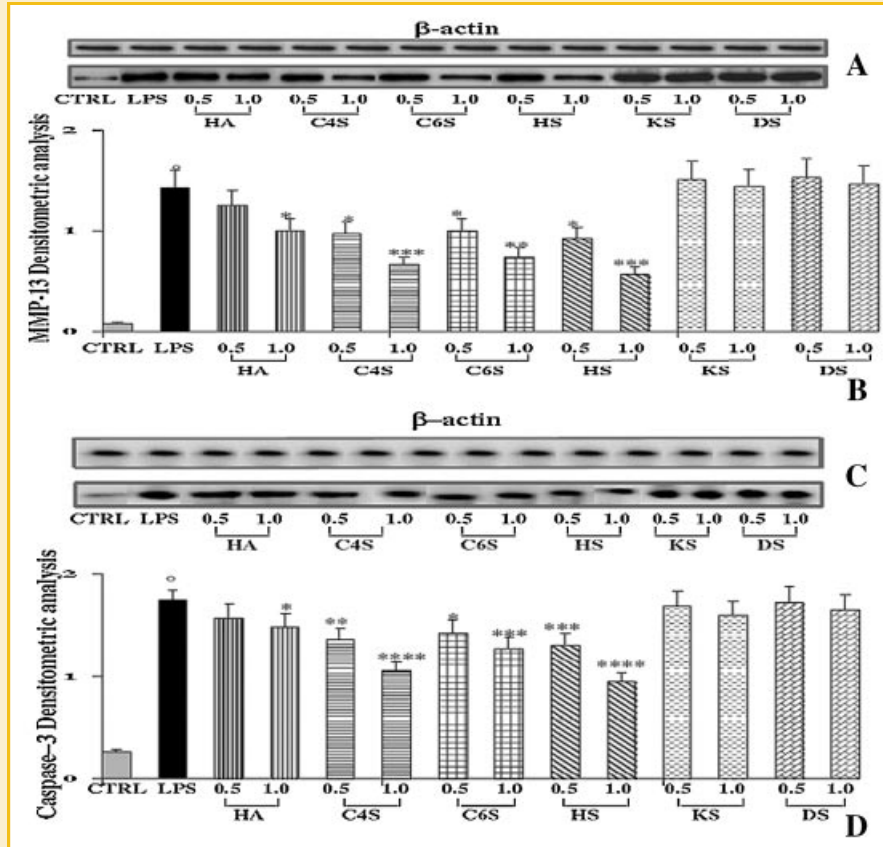
Fig. 4. Effect of GAG treatment on chondrocyte MMP–13 and caspase–3 protein production levels. GAGs were added to chondrocytes 4 h after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as both Western Blot analysis (panels A, C) and densitometric analysis (arbitrary units) (panels B, D). $°P < 0.001$ versus control; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ and $^{****}P < 0.001$ versus LPS.

initiator. The treatment with GAGs produced the following effects: at the higher dose HA only slightly decreased inflammatory cytokines, iNOS, MMPs and caspase-3; C6S significantly affected all parameters at both doses; C4S and HS were able to reduce protein production of inflammatory cytokines, iNOS, MMPs and the apoptotic initiator more significantly than C6S at both doses; KS and DS showed no positive reduction (Figs. 2–4). As TNF-α, IL-1β, IL-6, IFN-γ, iNOS, MMP-1, MMP-13 and caspase-3 mRNA expressions showed a similar pattern of protein levels the data were not reported.

DNA STRAND BREAKS BY TUNEL ASSAY

Nuclei of apoptotic chondrocytes stained brown whereas the negative cells stained blue. After treatment with LPS, chondrocytes DNA strand broke, and about 25% nuclei became condensed and tight. Examination of DAB stained cells has shown that the TUNEL positive cells consistently exhibited nuclear condensation with nuclear blebbing, both hallmarks of apoptosis. The treatment with GAGs confirmed the data obtained on caspase-3 inhibition and in particular produced the following effects: HA slightly protected chondrocytes with the highest dose only, C4S, C6S and HS provided significant protection to cells at both the doses used, although C6S

effect was less significant, and KS and DS did not exert any anti-apoptotic effect (Fig. 5).

NO PRODUCTION

Figure 6 shows the changes in NO levels of chondrocyte cultures after LPS stimulation and GAG treatment. A significant increase in NO release was seen in cells stimulated with LPS alone. The treatment with the GAGs exerted the following effects: HA, C4S and HS reduced NO generation at both doses; the other GAGs did not exert any reduction in NO release. In fact, although C6S was able to reduce significantly mRNA iNOS expression and protein synthesis, NO concentrations were not reduced. In addition, HA at the lower dose had no effect in reducing mRNA iNOS and protein production, while it was able to reduce NO levels significantly at the same dose (Fig. 6). In general for this parameter, the effects exerted by GAGs on mRNA iNOS and protein synthesis were not matched by NO generation.

NF–ᴋB ACTIVATION AND IKBα DEGRADATION

Figure 7 (panel A) shows the variations in the NF-κB p50/p65 heterodimer translocation throughout the experiment. LPS stimulation induced massive NF-κB translocation into the nucleus; GAG treatment showed the following effects: HA slightly decreased NF-
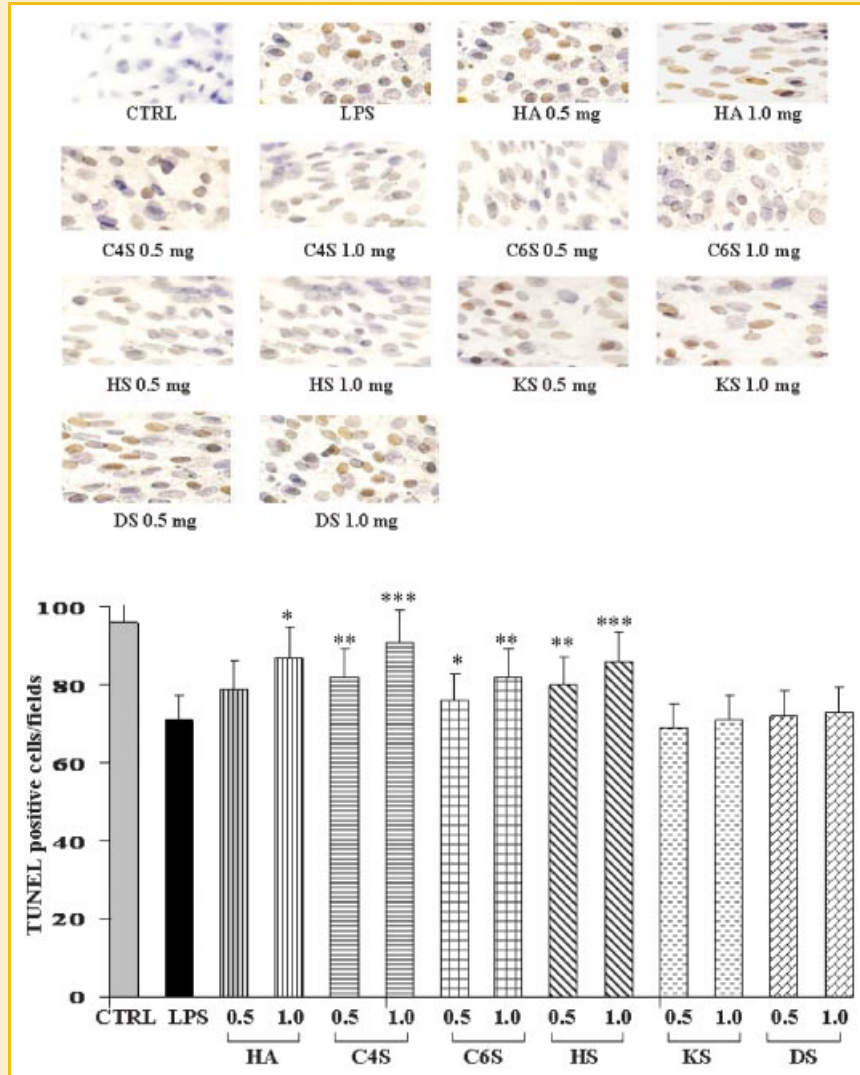
Fig. 5. Effect of GAG treatment on chondrocyte apoptosis. DNA strand fragmentation was evaluated by TUNEL assay technique. The positive (apoptotic) nuclei were stained brown and the negative (normal) were blue. GAGs were added to chondrocytes 4 h after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as percentages of apoptotic chondrocytes with respect the total chondrocytes. Magnification 200×. °$P < 0.001$ versus control; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$ versus LPS. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

κB activation but only at the high dose; both doses of C6S significantly blunted NF-κB activation; both doses of C4S and HS inhibited, NF-κB activation more significantly than C6S; KS and DS did not show any significant reduction.

Since IkBα protein is normally bound with NF-κB transcription factor, it was assayed in order to evaluate the degree of NF-κB activation. Figure 7 (panel B) shows that LPS stimulation induced a marked loss in IkBα protein. The changes in IkBα degradation exerted by HA, C4S, C6S and HS confirmed the results obtained for NF-κB activation.

DISCUSSION

The present study demonstrated that HA, C4S, C6S and HS were able to modulate LPS-induced inflammation in mouse articular chondrocytes. The effects were made evident by the improvement in cell survival, inhibition of NF-κB nuclear translocation, pro-inflammatory cytokine reduction, and diminution of both NO generation and caspase-3 activation. In contrast, KS and DS failed to reduce inflammation. The anti-inflammatory activity exerted by the active GAGs was more significant for C4S and HS, while HA at the lowest dose failed to exert any positive effect. C6S treatment also reduced pro-inflammatory cytokines, with both doses, but was less active than C4S and HS.

Pro-inflammatory cytokines, metalloproteases (MMPs), NO and other detrimental intermediates produced by stimulated cells play a critical role in inflammatory diseases such as sepsis and arthritis [Jean-Baptiste, 2007; Filer et al., 2008]. LPS-stimulated chondrocytes showed an inflammatory response by increasing TNF-α production, which in turn activates NF-κB translocation into the nucleus. The activation of NF-κB involves the phosphorylation of
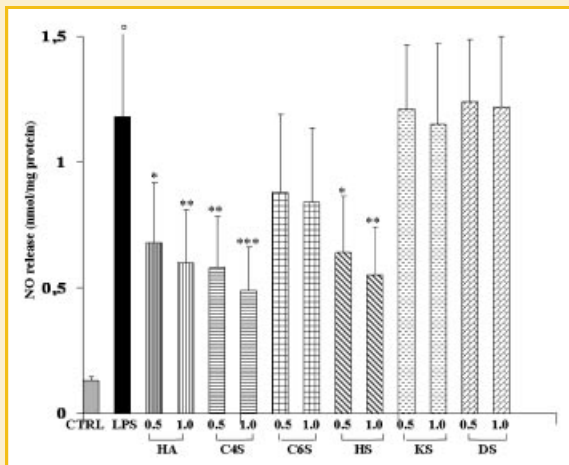
Fig. 6. Effect of GAG treatment on chondrocyte NO release. GAGs were added to chondrocytes 4 h after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as nmol/mg of protein. °$P < 0.001$ versus control; *$P < 0.05$, **$P < 0.01$ and $P < 0.005$ versus LPS.

IkBs via the IkB kinase (IKK) signalosome complex. The resulting free NF-κB is then translocated to the nucleus, where it binds to kB binding sites in the promoter regions of target genes, and induces the
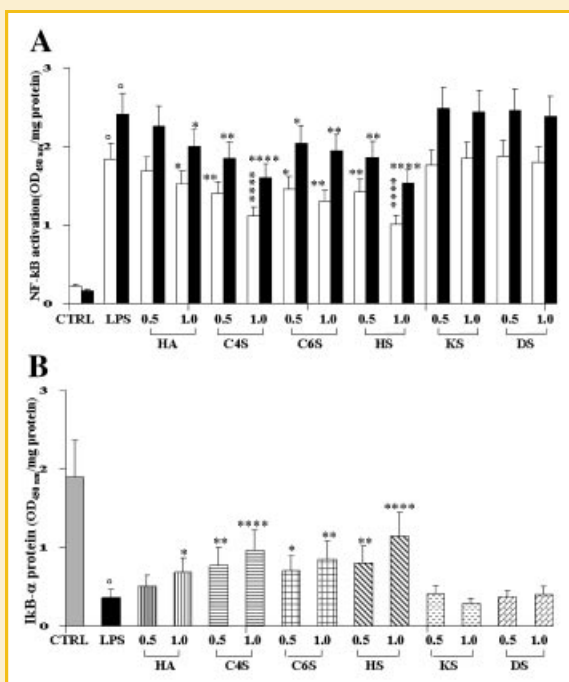

Fig. 7. Effect of GAG treatment on chondrocyte NF–κB p50/65 transcription factor DNA binding activity (panel A) and IkB–α protein degradation (panel B). GAGs were added to chondrocytes 4 h after LPS stimulation. In panel A, white bars represent the p/50 subunit; black bars represent the p/65 subunit. Values are the mean ± SD of seven experiments and are expressed as optical density at λ 450 nm/mg protein of nuclear extract (panel A) and as optical density measured at λ 450 nm/mg protein (panel B). °$P < 0.001$ versus control; *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ and ****$P < 0.001$ versus LPS.

transcription of pro-inflammatory mediators such as TNF-α, IL-1β, IFN-γ iNOS, MMPs and caspases [Baeuerle and Baltimore, 1996]. The activation of all these factors contributes to cell death and tissue disruption.

The interaction of cells with the surrounding extracellular matrix is essential in many physiological and pathological mechanisms. Proteoglycans may influence cell behaviour through binding events mediated by their GAG chains. The specificity of protein–GAG interactions is governed by the ionic attractions of sulphate and carboxylate groups of GAGs with the basic amino acids on the protein as well as the optimal structural fit of the GAG chain into the binding site of the protein [Yates et al., 2006]. The binding affinity of the interaction depends on the ability of the oligosaccharide sequence to provide optimal charge (orientation of sulphate groups) and surface (van der Waals contact) with the protein [Yates et al., 2006].

We previously reported that HA and C4S were able to reduce free radical damage in fibroblast cultures exposed to $FeSO_4$ plus ascorbate [Campo et al., 2008]. The actions of HA and C4S were also able to inhibit NF-κB and executioner caspase activation [Campo et al., 2008]. We hypothesized that the inhibition of NF-κB DNA binding to the nucleus may be the consequence of HA plus C4S reduced ROS production in the fibroblasts. Since ROS are able to activate these pathways [Gloire et al., 2006], then HA plus C4S treatment probably reduced the activation of NF-κB by preventing the oxidative burst. We also hypothesized that the same line of reasoning could be extended to apoptosis activation. However, direct inhibition of NF-κB cannot be excluded. In order to ascertain this effect, in this study we employed an LPS-induced inflammation in chondrocyte cultures in which there was no immediate and direct production of ROS, such as OH·, H_2O_2 or O_2^- [Haglund et al., 2008]. However, LPS produced a marked increase in NO levels through iNOS activation and this in turn may also stimulate ROS production [Wu et al., 2007]. The data obtained show that HA, C4S, C6S and HS may reduce pro-inflammatory cytokines, MMPs, NO and apoptosis through the inhibition of NF-κB translocation, although with different effects. The inhibitory effect on NF-κB activation exerted by active GAGs may be due to direct interaction with the nuclear factor due to the presence in its structure of a charged groups such as sulphated groups and carboxylic groups. These charged groups may interact with protein structures such as NF-κB with a consequent blocking of their activity and reduction in inflammation. Another explanation and molecular mechanism may be suggested, again due to the chemical structure of these charged polymers. Previous studies reported that certain chemokines, including pro-inflammatory cytokines, require interactions with GAGs for their in vivo function [Mulloy and Rider, 2006; Proudfoot, 2006]. This interaction is thought to play a role in the sequestration of chemokine and subsequent presentation to the receptor expressed on the leukocyte cell surface [Scott, 1992; Mulloy and Rider, 2006; Proudfoot, 2006]. It has been suggested that the unmatched inhibition of the interaction between pro-inflammatory cytokines such as IFN-γ and membrane-associated GAGs may provide a mechanism for inducing clinically useful immunosuppression [Douglas et al., 1997; Johnson et al., 2004]. In this context, it is conceivable that since NF-κB, just as it may induce pro-inflammatory cytokine production,

may be itself induced by pro-inflammatory cytokines, GAGs could inhibit pro-inflammatory cytokines that in turn inhibit NF-κB activation. In conclusion, GAG interaction may be directly involve pro-inflammatory cytokines or NF-κB or both. Therefore, the positive modulatory effect exerted by GAGs on all considered parameters may be due to its efficiency to bind protein structures thereby exerting an inhibitory activity.

In our study, HA was able to improve chondrocyte survival and to reduce NO levels in a dose dependent manner, although at limit of the threshold of significance. Furthermore the higher dose slightly reduced pro-inflammatory cytokines, iNOS, MMPs and caspase-3, while the lower dose failed to do so. Therefore, the lower HA dose reduced NO levels without any effect on iNOS mRNA expression and protein production. This paradox may be justified by the fact that HA possesses a free radical scavenger activity and consequently may also have directly bound NO at this low dosage, while an increased HA dose is necessary to inhibit NF-κB activation. C4S exerted a greater effect than HA, C6S and HS for all the parameters considered. This may be due to the presence in its structure of a sulphated group in position 4 opposite the carboxylic group. These charged groups may interact with NO or other protein structures such as NF-κB more strongly than the other GAGs, with a consequent blocking of their activity and reduction in inflammation. HS, instead, significantly inhibited the pro-inflammatory cytokines, iNOS, MMPs and caspase-3 production, and reduced NO levels. HS was also able to improve chondrocyte survival at both doses used. The positive effect of HS on inflammation has been extensively documented [Bartlett et al., 2007; Lindahl, 2007; Kliment et al., 2008]; the positive effects exerted on cell survival and the reduction of NO levels may be due to its free radical scavenger activity [Kolset and Salmivirta, 1999; Campo et al., 2004]. Due to their heterogeneity in sulphate distribution, HS chains interact with a multitude of proteins [Lindahl, 2007]. C6S had a significant effect on decreasing pro-inflammatory cytokines, iNOS, MMPs and caspase-3, although the effects were less evident than with C4S and HS, while it failed to exert any positive action on NO levels and on cell viability at the lower dose. This smaller effect, compared to C4S and HS, may be justified by the fact that C6S has the sulphated group in a peripheral position, and this may aggregate, while C4S should not form aggregates due to its sulphate groups being near the midline of the polymer [Scott, 1992]. The lack of effect on NO levels and on cell viability at the lower dose may be related to the low antioxidant activity of C6S [Campo et al., 2004]. KS and DS failed to exert any significant action on any of the parameters considered. The explanation for this absence of effects is probably due to their chemical structure [Iozzo, 1998], since in previous reports they have not shown any antioxidant activity [Campo et al., 2004], particularly KS [Albertini et al., 1996]. Both KS and DS are structures that are able to self-aggregate [Scott, 1992].

These results further support the hypothesis that circulating GAGs may function as immunomodulators and their increased release and degradation could be a biological response that acts to modulate inflammation during disease. Their different effects depend upon the different structural properties of their polysaccharide chain.

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