BioFactors

Research Communication

Hyaluronan differently modulates TLR-4 and the inflammatory response in mouse chondrocytes

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Abstract.

Hyaluronic acid (HA) may exert different action depending on its degree of polymerization. Small HA fragments induce proinflammatory responses, while highly polymerized HA exerts a protective effect in inflammatory pathologies such as rheumatoid arthritis. In both cases the toll-like receptor 4 (TLR-4) seems to be involved in the modulation of the inflammation process. The aim of this study was to investigate the influence of short HA oligosaccharides (HA 4-mers) and high molecular weight HA (HMWHA) in the inflammatory response in normal mouse chondrocytes. Messenger RNA and related protein levels were measured for TLR-4, tumor necrosis factor-alpha (TNF-alpha), interleukin-1beta (IL-1beta), interleukin-6 (IL-6), and interleukin-18 (IL-18) in cells with and without the addition of HA. NF-kB activation was also evaluated. 4-mer HA treatment produced a significant up-regulation of all parameters considered while HMWHA did not exert any activity in untreated cells although it was able to reduce the effects of 4- mers HA significantly. Specific TLR-4 small interference RNA (siRNA) was used to confirm TLR-4 as the target of HA action. This study suggests that HA may modulate proinflammatory cytokines via its different degree of polymerization and inflammatory action may be modulated as a result of the interaction between HA and TLR-4.

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1. Introduction

The altered assembly of connective tissue, resulting either from the synthesis or degradation of the extracellular matrix (ECM) components, can markedly modify cell–matrix interactions by activating various signaling pathways that regulate cell behavior [1]. The ECM is an important component of tissue microenvironment, which provides biophysical and biochemical cues that maintain the integrity of tissue architecture. Modification of these parameters appears to be an integral factor in promoting the onset and progression of inflammatory diseases such as rheumatoid arthritis (RA) [2].

The active ECM fragments are called matrikines and they may play various roles. Matrikines include those from collagen type one and four, elastin, fibronectin, laminins, entactin/nidogen, thrombospondin, and hyaluronan (HA) which may act as potent inflammatory mediators [3]. HA is the most widely studied of these because of its activity changes depending on its state of aggregation. HA is an abundant basic component of the ECM, mainly present as a high molecular mass polymer ($>10^6$ Da) [4]. High and low molecular weight forms of HA exhibit opposite effects on cell behavior. Extracellular HMWHA inhibits endothelial cell growth, and is therefore antiangiogenic in nature. HA polymers have a notable ability to bind fibrinogen, and thus play a critical role in wound healing [5]. HA polymers are also anti-inflammatory and immunosuppressive in nature [6]. Fetal circulation and amniotic fluid contain high concentrations of HMWHA, which may account for some of the immunosuppression in the developing fetus. Furthermore, production of HMWHA is increased at sites of inflammation, often correlating with leukocyte adhesion and migration. It has been found that peripheral blood monocytes are stimulated through their binding to HMWHA chains, but this interaction induces the expression of growth factors and matrix components rather than proinflammatory mediators [7]. These findings provide evidence for the anti-inflammatory role played by HMWHA. Recent studies have also reported that HMWHA is able to modulate TLR-4 both *in vitro* and

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in vivo [8,9]. Its polymeric structure seems able to mask active sites of TLR-4, thereby preventing the binding of these receptors with PAMPs.

Following tissue injury it has been shown that HA fragments of low molecular weight are produced as a result of hyaluronidase activity or oxidation [10–12]. In particular, HA has been shown to have greater polydispersity in size under inflammatory conditions, with a preponderance of low molecular weight forms [11,12]. Small HA fragments are also involved in a variety of normal and pathological processes [6]. HA oligosaccharides can exert many different effects in relation to their size. They interact with a different set of receptors that trigger signaling cascades and initiate profound changes in cell behavior. HA fragments have been shown to promote angiogenesis in several experimental models [13]. Moreover, they enhance the synthesis of types I and VIII collagen, which are ECM molecules of the endothelial cell angiogenic phenotype. HA oligosaccharides are potent stimulators of inflammatory cytokine and adhesion molecules [9,11,14–15]. Studies on activated macrophages have shown that HA fragments induce the expression of chemokines such as macrophage inflammatory protein-1a/b, RANTES and monocyte chemotactic protein-1, the functions of which are crucial in initiating and maintaining the inflammatory response [16]. Furthermore, expression of hyaluronan synthase-2, aggrecan, matrix metalloproteinase-3/13, and inducible nitric oxide synthase have been stimulated by small HA fragments in various types of chondrocytes [17,18]. Short HA fragments have been reported to promote cell proliferation of chondrocytes [18], endothelial cells and fibroblasts. Small HA oligomers (6–20 kDa size range) are potent activators of dendritic cells, the antigen-presenting cells of the immune system. Thus, HA fragments tend to be angiogenic, immuno-stimulatory, and inflammatory [11,19,20]. Very small HA oligosaccharides also have unique specific biological activities. Oligomers of six disaccharides promote differentiation of the endothelial cells induced in response to the angiogenic effect of larger HA fragments [21]. Tetra and hexasaccharides are predominant products of hyaluronidase-mediated degradation. Tetrasaccharides induce expression of heat shock proteins and are antiapoptotic, suppressing cell death in cultures subjected to hyperthermia [22]. Other studies have shown that hexasaccharides act as antagonists to HMWHA, interfering with normal bovine chondrocyte cell–matrix interactions such as pericellular matrix assembly [23].

Previous investigations have also shown that small fragments of HA or HA at low molecular weight can interact with toll-like receptor-4 (TLR-4), thereby stimulating inflammation or increasing the inflammatory mechanism previously induced by other agents in different cell types [8,9]. Thus, the generation of lower molecular weight HA in pathologies may act as an endogenous danger signal, leading to the activation of both innate and acquired immunity. HA fragments may prime inflammation via TLR-4 that finally stimulates NF-kB activation. NF-kB then translocates into the nucleus where it may prime the transcription of several inflammatory mediators. Inflammation mediators are in turn responsible for HA synthase induction, with a consequent increase in HA production that is in part deposited in tissues and in part degraded. The net result is the amplification and the perpetuation of tissue/organ inflammation [10,24].

Most proinflammatory HA fragments, including oligomers of four–six disaccharides, can signal through TLR-4 in various cell types [9,15,19,20]. Other studies have confirmed that HA oligomers require MyD88, and TLR-4 both *in vitro* and *in vivo* to initiate the inflammatory response in acute lung injury [11]. Hence, HA catabolism depends on individual hyaluronidase activities under pathological conditions and the products generated along the catabolic pathway are able to produce contrasting biological activities.

In this study, we investigated the influence of short HA oligosaccharides (HA-4 mers) and high molecular weight HA (HMWHA) on the inflammatory response in normal mouse chondrocytes. We also studied the effects on murine chondrocytes stimulated with HA-4 mers of adding a specific TLR-4 small interference RNA (siRNAs), which blocks TLR-4 expression.

2. Methods

2.1. Animals

Male mice DBA/J1 6-7 weeks old with a mean weight of 25–30 g were used in our study. Mice, purchased from Harlan (Correzzana, Italy), were maintained under climate-controlled conditions with a 12-h light/dark cycle. The animals were fed standard rodent chow and provided water *ad libitum*. The health status of the animal colony was monitored in accordance with Italian Veterinary Board guidelines.

2.2. Materials

HA 4-mer oligosaccharides as sodium salt (cat n. CSR-11006) were obtained from Cosmo Bio (Tokyo, Japan). High molecular weight HA (HMWHA) (4,000 KDa) (cat. N. H5388) was purchased from Sigma-Aldrich Srl (Milan, Italy). Both compounds were endotoxin free (endotoxin content $<$0.1 ng/mg). To confirm purity, HA sample batches were assayed spectrophotometrically. HA preparations were free of DNA and protein contamination as preparations showed no absorbance at 260 and 280 nm. TLR-4 siRNA (sc-40261 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse TNF-α cat. IB49688), IL-1 β cat. IB49700), and IL-6 (cat. 27768) commercial ELISA kits were provided by Immuno-Biological laboratories (Minneapolis, MN). Mouse IL-18 (cat. 7625) ELISA kits were purchased from R&D Systems (Minneapolis, MN). Mouse TLR-4 (cat. ABIN424269) commercial ELISA kits were provided by Antibodies-online.com GmbH, (Eachen, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, trypsin-EDTA solution and phosphate buffered saline (PBS) were obtained from Life Technologies Gibco/Brl Division (Grand Island, NY). All cell culture plastics were obtained from Falcon (Oxnard, CA).

RNase, proteinase K, protease inhibitor cocktail, sodium dodecylsulfate (SDS) and all other general laboratory chemicals were obtained from Sigma-Aldrich Srl. (Milan, Italy).

2.3. Chondrocyte cultures

Mice were killed by cervical dislocation and whole patellae were dissected from the hind knee joint. Chondrocytes were released from articular cartilage after being digested with 0.2% pronase for 1 h, followed by digestion in PBS containing 0.25% trypsin and 0.05% EDTA at 37°C for 1 h. After washing with PBS, cartilage was further digested for 3 h with PBS containing 0.1% collagenase in DMEM [25]. After removing undigested cartilage using a 70-μm nylon sieve, the chondrocytes were collected by centrifugation, washed with PBS, and cultured in 75 cm^2 plastic flasks containing 15 mL DMEM to which 10% FBS, L-glutamine (2.0 m*M*) and penicillin/streptomycin (100 U/mL, 100 μg/mL) were added. Cells were incubated at 37°C in humidified air with 5% CO_2. Experiments were performed using chondrocyte cultures between the third and the fifth passage.

2.4. Chondrocyte treatment

Chondrocytes were cultured in 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 either HA-4mers (40.0 μg/m), previous filtered using 0.22-μm filters, or HMWHA (200.0 μg/mL), or TLR-4 siRNA. Chondrocytes receiving TLR-4 siRNA plus HA-4mers and/or HMWHA were treated 48 h before HA-4mers and/or HMWHA, in order to block TLR-4 mRNA activity. In chondrocytes receiving both HA-4mers plus HMWHA, HMWHA was added 5 min after HA-4mers treatment. The cells and medium underwent final biochemical evaluation 24 h after the last treatment. The study therefore included the following groups of cells: (1) CTRL; (2) HA-4mers; (3) HMWHA; (4) HA-4mers + HMWHA; (5) TLR-4 siRNA; (6) TLR-4 siRNA + HA-4mers; (7) TLR-4 siRNA + HMWHA; (8) TLR-4 siRNA + HA-4mers + HMWHA.

2.5. siRNA treatment

For siRNA experiments, 4×10^6 cells were transfected with 4.0 μg TLR-4 siRNA in accordance with a modified siRNA Transfection Protocol (Santa Cruz Biotechnology, Santa Cruz, CA). Scramble siRNA was used under the same conditions as a negative control. After transfection, cells were seeded in 6-well plates in 10% FCS/DMEM and allowed to adhere for 12 h. Culture medium was then replaced with fresh 10% FCS/DMEM for a further 12 h. Cells were serum starved for 24 h before the addition of HA-4mers and/or HMWHA.

2.6. RNA isolation, cDNA synthesis, and real-time quantitative PCR amplification

Total RNA was isolated from chondrocytes for reverse-PCR real time analysis of TLR-4, TNF-α, IL-1 β, IL-6, and IL-18 (RealTime PCR system, Mod. 7500, Applied Biosystems, Carlsbad, CA) 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, Carlsbad, CA). β-actin mRNA was used as an endogenous control to allow the relative quantification of TLR-4, TNF-α, IL-1 β, IL-6, and IL-18. PCR RealTime was performed on both targets and endogenous controls by means of ready-to-use assays (Assays on demand, Applied Biosystems Inc, Carlsband, CA). The amplified PCR products were quantified by measuring the calculated cycle thresholds (C_T) of TLR-4, TNF-α, IL-1 β, IL-6, IL-18, and β-Actin mRNA. The C_T values were plotted against the log input RNA concentration in serially diluted total RNA of synovial fibroblast samples and used to generate standard curves for all mRNAs analyzed. The amounts of specific mRNA in samples were calculated using the $\Delta\Delta C_T$ method. The mean value of normal cartilage 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).

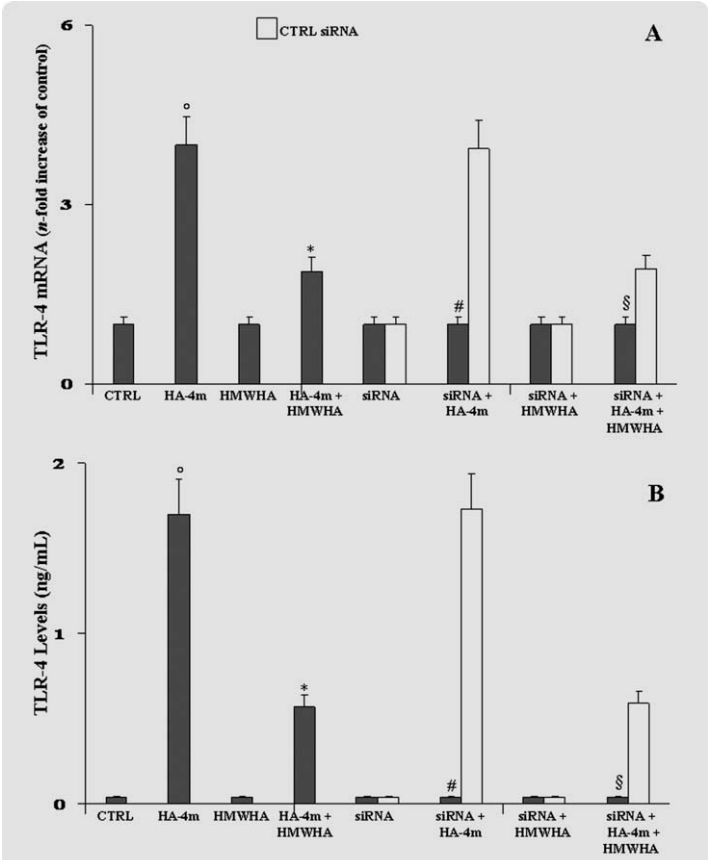

Fig. 1. Effect of HA-4mers, HMWHA, and TLR-4 siRNA treatment on mouse articular chondrocytes TLR-4 mRNA expression (A) and related protein production (B). White bars represent control siRNA. Values are the mean ± S.D. of no less than seven experiments and are expressed as the *n*-fold increase with respect to controls (A) and as ng/mL (B) for the TLR-4 protein levels. °$P < 0.001$ vs control; *$P < 0.001$ vs HA-4mers; #$P < 0.001$ vs HA-4mer; §$P < 0.001$ vs HA-4mer + HMWHA.

2.7. 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-kB activation. Analysis was performed in line with the manufacturer's protocol for a commercial kit (NF-kB p50/65 EZ-TFA Transcription Factor Assay Colorimetric, cat. n°70–510, Millipore Corp. Billerica, MA). About 4–5 $\times$ 10^5 chondrocytes were used for NF-kB assay. Cytosolic and nuclear separation was performed by lysing the cell membrane with an apposite hypotonic lysis buffer (20 m*M* Tris-HCl pH 7.5, 150 m*M* NaCl, 1.0 m*M* EDTA, 1.0 m*M* EGTA, 1.0% Triton, 2.5 m*M* $Na_4P_2O_7$. 1.0 m*M* β-glycerophosphate, 1.0 m*M* Na_3VO_4) containing protease inhibitor cocktail and tributylphosphine (TBP) as reducing agent. After centrifugation at 8,000*g*, the supernatant containing the cytosolic fraction was stored at −70°C, while the pellet containing the nuclear portion was resuspended in the apposite extraction buffer (25 m*M* HEPES pH 7.5, 500 m*M* NaCl, 1.0 m*M* DTT, 10 m*M* NaF, 10% Glycerol, 0.2% NP40, 5.0 m*M* $MgCl_2$) and the nuclei were disrupted by a series of drawing and ejecting actions. The nuclei suspension was then centrifuged at 16,000*g*. The supernatant fraction was the nuclear extract. After the determination of protein concentration, this extract was stored in aliquots at −80°C for the subsequent NF-kB assay. After incubation with primary and secondary antibodies, color 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)/mg protein.

2.8. TLR-4, TNF-α, IL-1β, IL-6, and IL-18 ELISA assay

Samples of protein extracted from the cell culture in the presence of protease inhibitor cocktail were first lysed using a specific lysing buffer, and then centrifuged at 10,000*g* at 4°C for 10 min. The analysis of TLR-4, TNF-α, IL-1 β, IL-6, and IL-18 was carried out using specific commercial kits in line with the manufacturer's protocol. TLR-4 values are expressed as ng/mL, while TNF-α, IL-1 β, IL-6, and IL-18 values are expressed as pg/mL.

2.9. Protein analysis

The amount of protein was determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA, USA) with bovine serum albumin as a standard in accordance with the published method [26].

2.10. 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 $P < 0.05$.

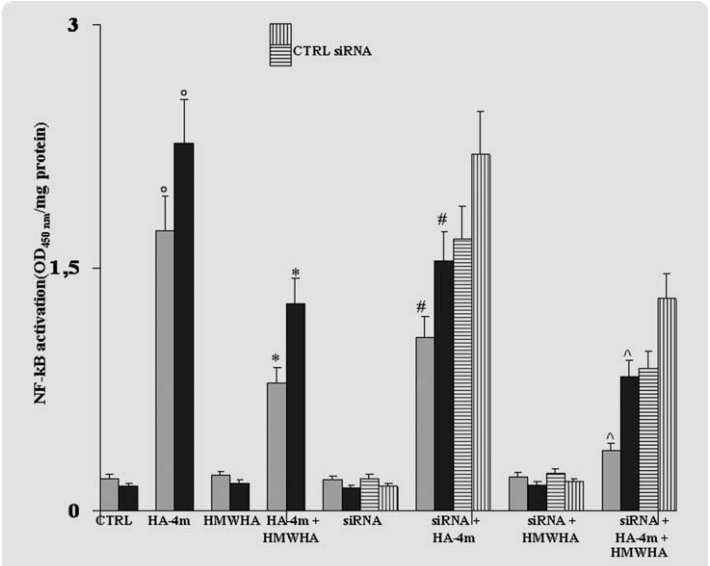

Fig. 2. Effect of HA-4mers, HMWHA, and TLR-4 siRNA treatment on mouse articular chondrocytes NF-kB p50/65 transcription factor DNA binding activity. Gray bars represent the p/50 subunit, black bars represent the p/65 subunit. Bars with horizontal lines and bars with vertical lines represent control siRNA, p/50 and p/65, respectively. Values are the mean ± S.D. of no less than seven experiments and are expressed as optical density at λ 450 nm/mg protein of nuclear extract. °$P < 0.001$ *vs.* control; *$P < 0.001$ *vs.* HA-4mers; #$P < 0.001$ *vs.* HA-4mers or *vs.* TLR-4 siRNA; ^$P < 0.001$ *vs.* TLR-4 siRNA + HA-4mers or *vs.* TLR-4 siRNA + HMWHA.

2.11. Statement of animal care

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

3. Results and discussion

We recently reported the ability of small HA oligosaccharides to stimulate inflammation in normal human chondrocytes [15]. In these studies it was shown that cytokines and other proinflammatory mediators were produced as the consequence of the interaction between either HA-6mers and TLR-4 or HA-6mers and CD44 receptors. Although HA stimulation of TLR-4 and CD44 receptors activated two distinct pathways, in the end they both converged in NF-kB activation [15]. The aim of this study was to evaluate TLR-4 involvement in chondrocytes following treatment with well-defined very small HA fragments, HA-4mers, and/or with HMWHA, previously shown to have a great capacity to modulate inflammation through these receptors [9,15,19,22,27–29].

In this study, we examined the effects of HA-4mers and HMWHA on the TLR-4 modulation in normal mouse articular chondrocytes. We also examined the effect of the

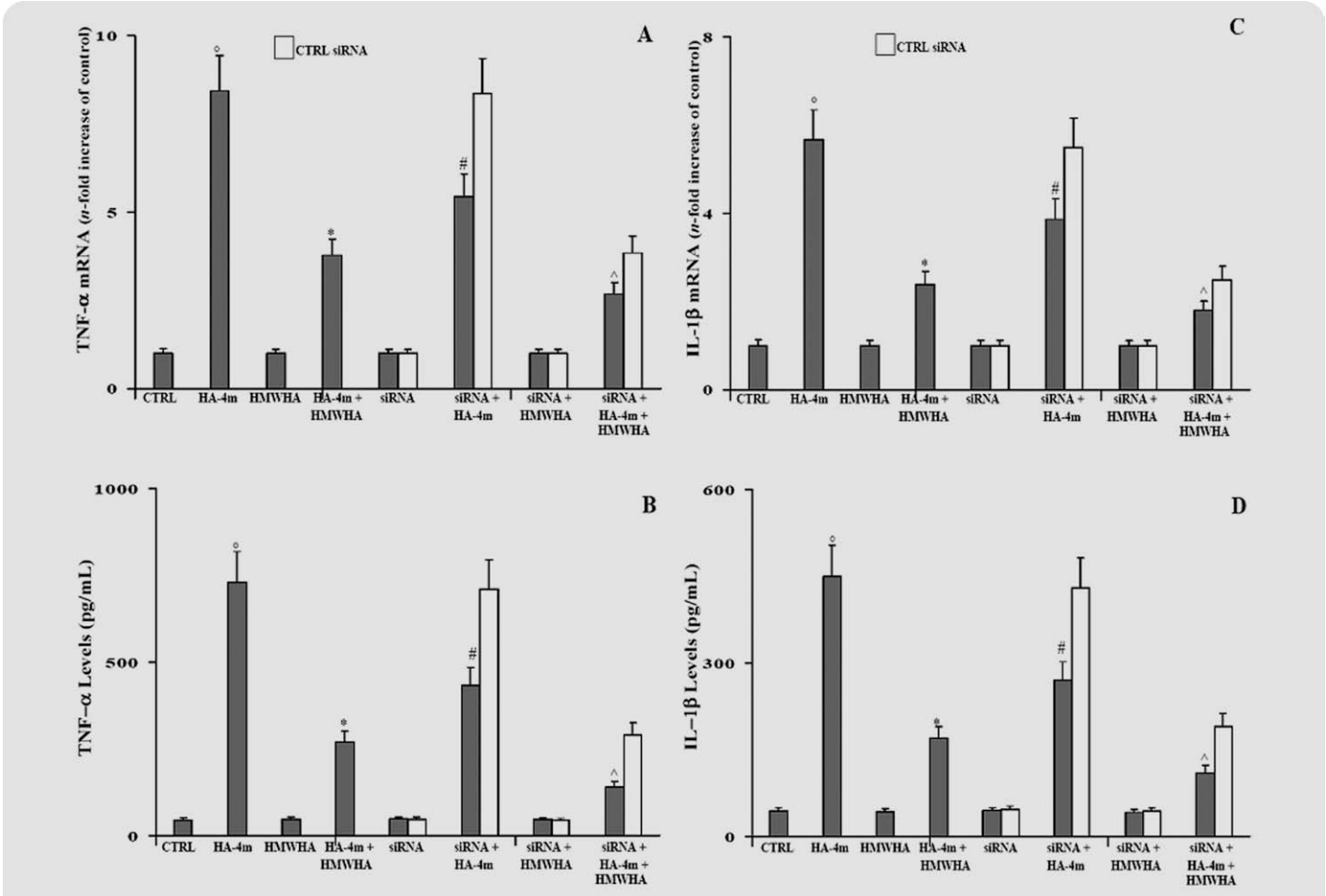

Fig. 3. Effect of HA-4mers, HMWHA, and TLR-4 siRNA treatment on mouse articular chondrocytes TNF-α and IL-1 β mRNA expression (A and C, respectively) and related protein production (B and D, respectively). White bars represent control siRNA. Values are the mean ± S.D. of no less than seven experiments and are expressed as the *n*-fold increase with respect to Controls (A and C) and as pg/mL (B and D) for the TNF-α and IL-1 β protein levels. °$P < 0.001$ *vs.* control; *$P < 0.001$ *vs.* HA-4mers; #$P < 0.001$ *vs.* HA-4mers or *vs.* TLR-4 siRNA; ^$P < 0.001$ *vs.* TLR-4 siRNA + HA-4mers or *vs.* TLR-4 siRNA + HMWHA.

pre-treatment of these group with a specific TLR-4 siRNA that blocks TLR-4 expression.

TLR-4 mRNA evaluation (Fig. 1A) and the related protein evaluation (Fig. 1B) were assayed in order to estimate the degree of TLR-4 activation in the presence of HA-4mers, and/orHMWHA, and/or TLR-4 siRNA. The data showed a marked expression and protein synthesis of the TLR-4 in chondrocytes treated with HA-4mers. In chondrocytes receiving both HA-4mers and HMWHA, however, a significant reduction in TLR-4 expression exerted by HMWHA occurred. Conversely, in chondrocytes pretreated with TLR-4 siRNA, the addition of HA-4mers failed to produce any increase in TLR-4 expression, since its expression had been blocked. The same results were obtained in all groups of chondrocytes pretreated with TLR-4 siRNA. This could be explained by the evidence found of HA-4mers being able to stimulate TLR-4 expression by stimulating the receptor, while HMWHA was able to reduce this stimulation by masking active sites of TLR-4, thus impeding the HA-4mers-TLR-4 binding. In contrast, TLR-4 siRNA abolished the HA-4mers effect, by blocking TLR-4 expression. Therefore, these data suggest that the degraded HA produced during inflammatory diseases is able to activate TLR-4 expression, while native HA acts by impeding TLR-4 stimulated by fragmented HA or other pathogen-associated molecular pattern (PAMPs).

Figure 2 shows the changes in the NF-kB p50/p65 heterodimer translocation over the course of the experiment in chondrocytes treated with HA-4mers, and/or HMWHA, and/or TLR-4 siRNA. This assay was also carried out in order to estimate the onset of inflammatory process, since the NF-kB factor can be activated by the TLR-4 pathway that in turn may converge to stimulate the expression of several genes that prime/amplify inflammation. For NF-kB we found a significant increase in its activation in chondrocytes treated with HA-4mers. In chondrocytes receiving both HA-4mers and HMWHA, a significant reduction in NF-kB translocation

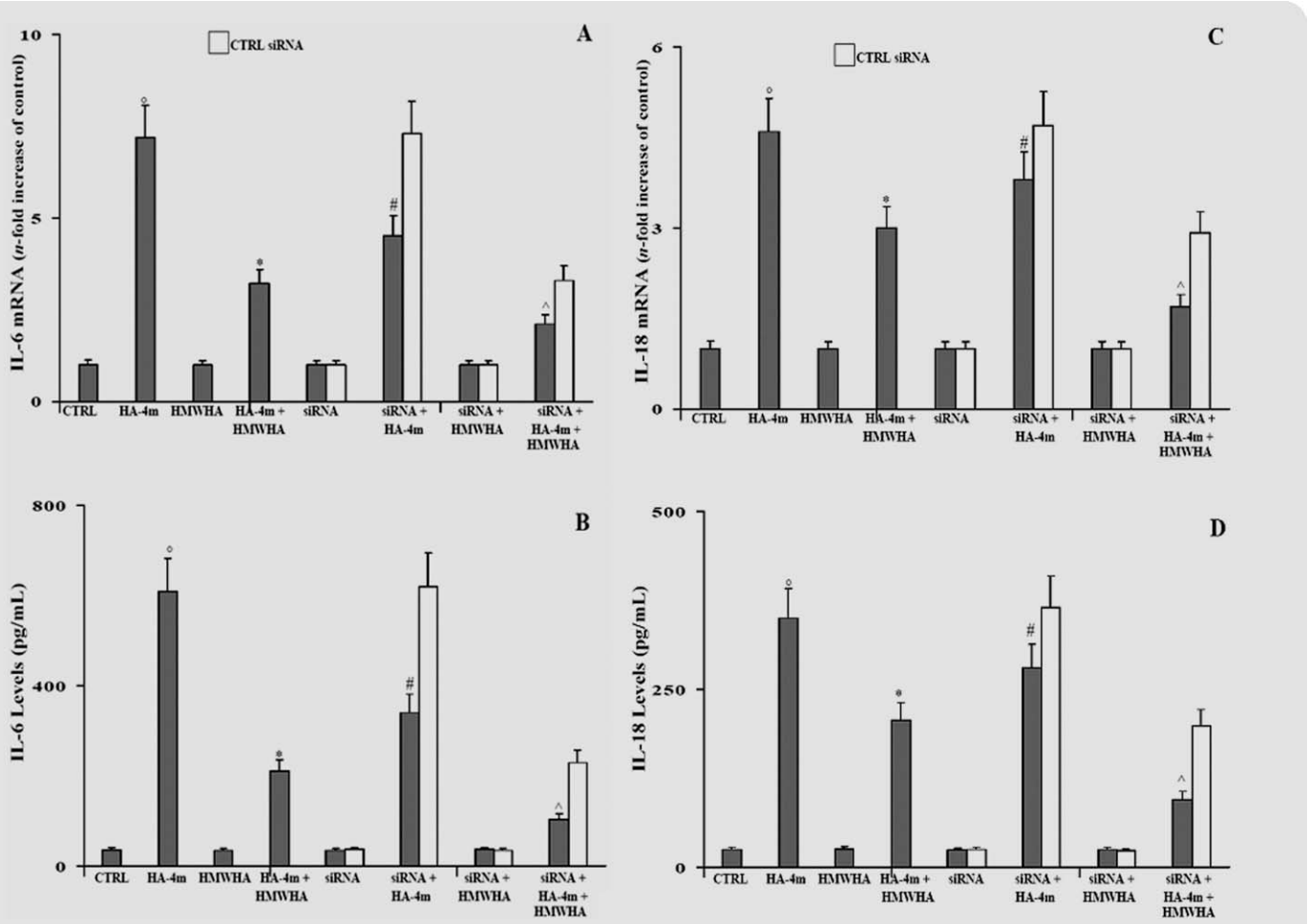

Fig. 4. Effect of HA-4mers, HMWHA, and TLR-4 siRNA treatment on mouse articular chondrocytes IL-6 and IL-18 mRNA expression (A and C, respectively) and related protein production (B and D, respectively). White bars represent control siRNA. Values are the mean ± S.D. of no less than seven experiments and are expressed as the *n*-fold increase with respect to controls (A and C) and as pg/mL (B and D) for the IL-6 and IL-18 protein levels. °$P < 0.001$ *vs.* Control; *$P < 0.001$ *vs.* HA-4mers; #$P < 0.001$ *vs.* HA-4mers or *vs.* TLR-4 siRNA; ^$P < 0.001$ *vs.* TLR-4 siRNA + HA-4mers or *vs.* TLR-4 siRNA + HMWHA.

was observed with respect to that observed for the addition of HA-4mers alone. The results obtained by evaluating the NF-kB factor in TLR-4 siRNA-treated chondrocytes plus HA-4mers confirmed that fragmented HA was able to activate TLR-4-mediated NF-kB expression. In chondrocytes receiving TLR-4 siRNA plus HA-4mers, the increment in NF-kB activation was significantly reduced, but not abolished. This could mean that the activation of NF-kB is not only due to TLR-4 stimulation exerted by HA-4mers but also by other HA-activated receptors. Interestingly, the addition of HMWHA to chondrocytes receiving both TLR-4 siRNA and HA-4mers was able to further reduce NF-kB expression. This could be explained by the fact that HMWHA may bind/mask other HA-4mers-activated-structures responsible for NF-kB activation. These findings confirm that degraded HA is able to activate NF-kB translocation through TLR-4 interaction.

TNF-α (Fig. 3, panel A), IL-1β (Fig. 3, panel C), IL-6 (Fig. 4, panel A), and IL-18 (Fig. 4, panel C) mRNA evaluation, and ELISA assay (panels B and D of each Figure) confirmed the previous data obtained by evaluating the NF-kB factor. In fact, the results showed a marked increase in the expression and protein synthesis of all inflammatory parameters in chondrocytes treated with HA-4mers. This increase may be explained as a direct consequence of NF-kB activation due to the TLR-4 stimulated by HA-4mers. No effects were seen when treating chondrocytes with HMWHA or TLR-4 siRNA alone. The addition of HMWHA to chondrocytes stimulated with HA-4mers was able to reduce the levels of all the inflammatory cytokines considered. Chondrocytes receiving both TLR-4 siRNA and HA-4mers showed a marked decrease in these inflammatory parameters since TLR-4 expression was abolished. However, cytokine expression was not fully abolished, as previously reported for NF-kB activation; other inflammatory pathways could be activated by fragmented HA, and therefore by HA-4mers to stimulate cytokine expression. HMWHA significantly reduced

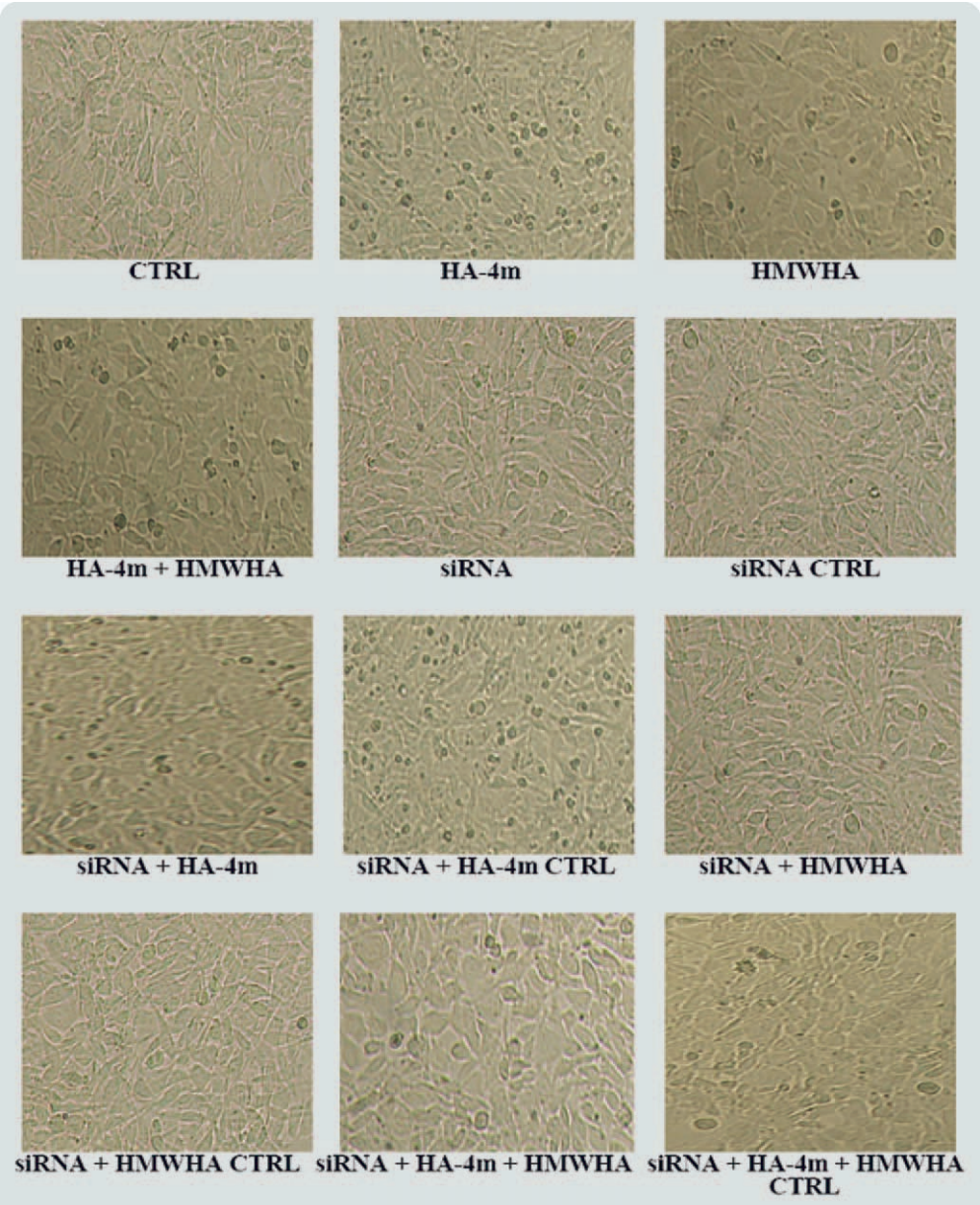

Fig. 5. Effect of HA-4mers, HMWHA, and TLR-4 siRNA treatment on mouse articular chondrocyte viability and morphology. (magnification, ×40). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

inflammatory cytokines in cells treated with HA-4mers alone as well as in those pretreated with TLR-4 siRNA and then with HA-4mers. These findings clearly imply that HMWHA may act on other inflammation mediator structures stimulated by HA-4mers- in addition to TLR-4. These results confirm the inflammatory role of degraded HA as mediator, and the anti-inflammatory role of native HA, as blocking agent, during the complex mechanism of inflammation.

In conclusion, as both native and degraded HA are able to bind a wide range of biological molecules, the stimulation/blocking of TLR-4 during inflammation needs to be taken into account in order to better clarify the inflammatory mechanism. Furthermore, we believe that the HA pathways should be carefully considered for future anti-inflammatory strategies, although further studies are needed to fully confirm this complex mechanism.

Acknowledgements

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