The biological action of hyaluronan on human osteoartritic articular chondrocytes: The importance of molecular weight

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Abstract

Objectives

The intra-articular injection of hyaluronan (HA) was originally used in the treatment of osteoarthritis (OA) to increase the viscosity of synovial fluid. However, some findings suggest that the activity of HA cannot be solely explained by its biomechanical properties. The aim of this study was to analyze the in vitro *biological effects of HA on human OA chondrocytes and the impact of its molecular weight (MW) on those effects.*

Methods

Cells were isolated from cartilage obtained during joint replacement surgery in OA patients. The chondrocytes were cultured for 24 hours to detect prostaglandin E2 (PGE2) and for 48 hours to measure nitric oxide (NO), after which they were pre-incubated with HA and stimulated with interleukin-1 (IL-1) at 5 ng/ml. Two commercial HA preparations with different MWs were used: Hyalgan® (500-730 kDa, HA, Bioibérica S.A.) and Synvisc® (hylan of 6,000 kDa, Biomatrix Inc). NO was detected by the Greiss reaction and PGE2 was quantified by a commercial EIA in the supernatant. Apoptosis was induced by an NO donor (sodium nitroprusside, SNP) and the effect of HA on apoptosis was quantified by flow cytometry.

Results

Neither HApreparation studied had any effect on the basal production of NO or PGE2. However, the 500-730 kDa HA at 200 μg/ml reduced the synthesis of both IL-1-induced NO and PGE2 by 70% and 45% respectively. Furthermore both HA preparations at 200 μg/ml decreased the apoptosis induced by SNP, 500-730 kDa to 40% and 6,000 kDa to 36%.

Conclusion

HA may induce biological effects in addition to acting as a viscoelastic substance. This study suggests that HA preparations are different due to differences in biological activity resulting from MW.

Key words

Chondrocytes, nitric oxide, apoptosis, hyaluronan, osteoarthritis, molecular weight.

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Introduction

Hyaluronan (HA) is a heteropolysaccharide formed by a variable number of repeating units of D-glucuronic acid and N-acetylglucosamine. It belongs to the glycosaminoglycan family (1). Synoviocytes, fibroblasts and chondrocytes all synthesize HA, which is present in the synovial fluid and the extracellular matrix of cartilage (2, 3). HAis viscoelastic and behaves like a viscous liquid at low shear rates and like an elastic solid at high shear rates.

HA, which was previously thought to play only a structural role in maintaining the architecture of the extracellular matrix (ECM) (4), has a variety of effects on cell migration and proliferation *in vitro* (5). Some of these effects are mediated through cell surface receptors, three of which have been molecularly characterized, namely CD44, CD54 and RHAMM (4, 6). The binding of the HA ligand to its receptor(s) triggers signal transduction events that can direct cell trafficking during physiological and pathological events (4-7). In addition to the cell surface receptors, intracellular HA binding proteins have been described. These observations, together with the reported intracellular location of HA, point to additional novel mechanisms by which HA may regulate cell behavior (4).

In osteoarthritis (OA), the concentration of synovial fluid HA is reduced, the length of the chains are decreased, and the viscoelastic properties of the fluid are compromised (8,9). In the treatment of OA, intra-articular injection of HA is used to reduce joint pain (3,10-12). The original rationale for the use of this intra-articular injection of HAin OA was to increase the viscosity of the synovial fluid. The observation that the clinical results exceed the life span of the HA exogenously administered into the joint supports the view that effects other than the biomechanical properties of this molecule could explain its therapeutic effectiveness (3, 12).

The cellular effects of HA may explain the carry-over clinical results observed with the intra-articular injection of HA. Some of the reported *in vitro* biological effects of HA are: 1) inhibition of prostaglandin E2 (PGE2) and nitric oxide (NO) synthesis induced by interleukin-1 (IL-1); 2) protection against proteoglycan depletion; 3) protection against cytotoxicity induced by oxygen-derived free radicals and against apoptosis induced by NO and Fas stimulation; 4) modulation of leukocyte adherence, proliferation, migration and phagocytosis; and 5) suppression of cartilage matrix degradation by fibronectin fragments (3, 13-16).

Recently, it has been recognized that HA fragments (<200 kDa), but not native HA, induce inflammatory gene expression (iNOS, chemokines and IL-8) and activate the transcriptional regulator nuclear factor kappaB (NF-kappaB) (5, 17). These data support the hypothesis that some of the cellular effects of HA fragments are dependent on their molecular weight (MW) (5,11,17,18).

Several HA preparations with different MWs (between 500 to 6,000 kDa) have been produced commercially to treat human OA. The significance of the MW of injectable therapeutic HA has not been studied in great detail. The aim of this study was to analyze the importance of the MW of HA on its biological effects. Herein, we report that commercially available HA preparations with different MWs have different biologic effect profiles on human articular chondrocytes.

Materials and methods

Tissue source, chondrocyte isolation and culture

Human cartilage was obtained from the femoral heads of 9 OA patients undergoing joint replacement surgery, who had macroscopically fibrillated cartilage (mean age 66.4 ± 5 years). Fibrillated as well as normal-appearing cartilage from the OA joints was used for cell isolation. Longitudinal slices of cartilage were cut from tissue and tritured using a scalpel. The cartilage pieces were incubated at 37ºC with trypsin for 10 minutes, after which the trypsin solution was removed. Collagenase type IV (2mg/ml) (Sigma Chemical Co., St. Louis, MO) was then added and incubated at 37ºC for 12 hours. Human chondrocytes were recovered by centrifugation and counted using a Neubauer haemocytometer. The isolated chondro-

cytes (4×10^6 cells) were cultured in 162 cm^2 flasks (Costar, Cambridge, MA) in DMEM medium (Life Technologies, Paisley, Scotland, UK) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% glutamine and 10% fetal bovine serum (FBS) (Life Technologies). Chondrocytes were incubated at 37ºC in a humidified gas mixture containing 5% CO_2 balanced with air. In order to ensure the chondrocytic phenotype, only high density primary passage cells were used. Cell viability was assessed by trypan blue dye exclusion; stained cells were discarded before carrying out experiments.

General experimental procedures

Chondrocytes were cultured, as described above (19), in DMEM medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% glutamine and 5% FBS with the addition of IL-1 (5ng/ml) and/or HA (10- 200 µg/ml). Two HA preparations with different MWs were used: Hyalgan® (500-730 kDa, Bioibérica S.A.) and Synvisc ® (6,000kDa, Biomatrix Inc), at 10 µg/ml; 50 µg/ml; 100 µg/ml; 150 µg/ml and 200 µg/ml.

NO quantification: Chondrocytes were plated at 50,000 cells per well in 96-well plates and cultured for 48 hours, after which the culture supernatants were collected for the nitrite measurements. NO formation was detected by NO_2^- accumulation in the culture supernatants by the Greiss reaction using sodium nitrite as the standard (20). Briefly, 50 µl of culture supernatant was incubated with 50 µl of 1% sulfanilamide and 0.1% N-1-naphthylethylenediamide dihydrochloride in 5% H_3PO_4 at room temperature for 5 minutes. The optical density was then measured at 570 nm. The unstimulated (basal) and IL-1 stimulated production of NO were quantified in the supernatant.

Measurement of PGE2 levels: The conditioned media from chondrocytes at 75,000 cells/well in 96-well plates in 0.1 ml of medium were collected after 24 hours and stored at -80ºC (21). The PGE2 content was measured by an enzyme immunoassay (EIA) (Amersham Pharmacia Biotech), according to the manufacturer's instructions. The sensitivity of the assay was 40 pg/ml. The unstimulated (basal) and IL-1 stimulated production of PGE2 were quantified in the supernatant.

Analysis of chondrocytic apoptosis: Apoptosis was induced by a NO donor and the effect of HA on apoptosis was quantified by flow cytometry (22). Briefly, chondrocytes (500,000 cells/well in 6-well plates) were incubated with SNP at 2 mM added to the two HA preparations at different concentrations for 24 hours. Then, cells were fixed in 70% ethanol at 4ºC for 60 minutes, washed and incubated with RNAse (50 µg/ml) and propidium iodide (PI, 100 µg/ml) for 15 minutes at room temperature in the dark, and kept at 4ºC. The resulting PI fluorescence of the nuclei was measured by flow cytometry on a FACScan (Becton and Dickinson, Mountain View, CA) using a 560 nm dichromatic mirror and a 600 nm band pass filter. Data are expressed as the percent of apoptotic (hypodiploid) nuclei.

In each experiment, both HA preparations were tested using cells in triplicate cultures from the same donor; cells from different donors were never pooled. Cells from the same donor were employed to carry out a single experiment to test the effects of both HA preparations on NO or PGE2 synthesis or apoptosis. Six single assays were performed for each parameter studied.

Hyaluronan preparations employed

Hyalgan® is a highly purified preservative-free HA in phosphate buffered saline (PBS; 10 mg/ml) obtained from rooster combs and having a MW of 500-730 kDa. This HA, prepared following the rules approved by the European Pharmacopoeia, was kindly supplied by Bioiberica S.A. (Spain). The other HA used was Synvisc® (hylan G-F 20), a highly purified formulation of rooster comb HA. It is a high MW (6,000 kDa) crosslinked HAmarketed by Biomatrix Inc. in Spain. Solutions of both HApreparations were diluted with PBS to reach the desired working concentrations.

Data analyses

Results of the effect of HAon basal NO and PGE2 production are expressed as the mean ± SEM of six individual experiments. Results of the effect of HA on IL-1-induced NO and PGE2 levels are expressed as a percentage of the levels induced by IL-1 (levels of NO or PGE2 induced by IL-1 + HA/ levels of NO or PGE2 induced by IL-1 x 100). Results of the effect of HA on apoptosis were calculated as follows: % apoptosis induced by SNP+ HA/ % apoptosis induced by SNP x 100. Statistical analyses were performed with the unpaired two-tailed Student's t-test.

Results

Effect of HA preparations on inflammatory mediators produced by human articular chondrocytes

Neither HA preparation modified the basal NO levels (Table I). However, we found differences between the 500-730 kDa HAand the 6,000 kDa HA on NO synthesis induced by IL-1. While the 6,000 kDa HA did not alter the levels of NO produced by IL-1, the 500-730 kDa HA at concentrations higher than 100 µg/ml reduced NO production by 70% (Fig.1). Similar results were obtained when PGE2 synthesis was studied. Neither HA modified the basal synthesis of PGE2 (Table I). However, the 500-730 kDa HAdecreased the levels of PGE2 induced by IL-1 in a dose-dependent manner (Fig. 2).

Effect of HA preparations on chondrocyte apoptosis induced by nitric oxide

Based on our previous findings, SNPat 2 mM was employed to induce chondrocyte apoptosis (23). The NO donor increased apoptosis to a value of 87 ± 5% after 24 hours (data not shown). However, the incubation of SNP with different concentrations of both HA preparations reduced apoptosis in a dose-dependent manner. At 200 µg/ml, the 6,000 kDa HA decreased apoptosis by 36% and the 500-730 kDa HAdecreased apoptosis by 40% (Fig. 3). This effect was significant with respect to the percentage of apoptosis induced by SNP($p < 0.05$)

Discussion

This study supports the concept that the MW of HA plays an important role in the biological effects of HA on human OA articular chondrocytes These find-

Table I. Effect of hyaluronan on basal synthesis of NO and PGE2.

Stimuli	NO (Mean ± SD) (µmoles nitrites)		PGE2 (Mean ± SD) (pg/ml)	
Basal	2.9 ± 1.1		270 ± 50.1	
IL-1 (5 ng/ml)	124 ± 20.5		721 ± 94.3	
	MW 570-730 kDa	MW 6,000 kDa	MW 570-730 kDa	MW 6,000 kDa
HA10 µg/ml	2.8± 1.2	3 ± 1.3	260.5 ± 41.2	284.7 ± 44.3
HA50 µg/ml	3.0± 1.6	2.7 ± 1.5	283.1 ± 43.5	279.3 ± 45.1
HA100 µg/ml	3.3± 1.4	3 ± 1.3	277.3 ± 39.9	288.4 ± 47
HA150 µg/ml	3.4± 1.5	3.4 ± 1.4	281.2 ± 40.1	290.1 ± 48.7
HA200 µg/ml	3.6± 1.9	3.6 ± 1.7	285.1 ± 45.6	296.3 ± 50.5

NO: Nitric oxide; PGE2: Prostaglandin E2; IL-1: Interleukin-1; HA: Hyaluronan; kDa: kilodaltons; MW: molecular weight.

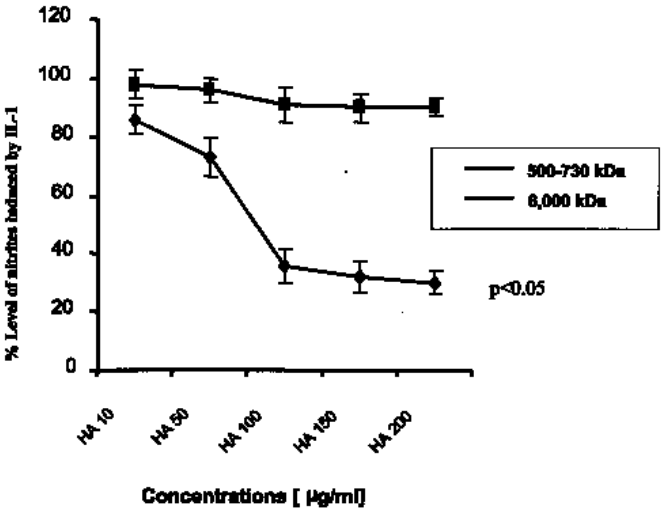

Fig. 1. Effect of two HApreparations on IL-1 induced synthesis of NO. OAcells were seeded into the well (50,000 cells/well in a 96-well plate) with 0.1 ml of medium and incubated with IL-1 at 5 ng/ml for 48 hours. Different concentrations (10-200 µg/ml) of two HApreparations (500-730 kDa and 6000 kDa) were simultaneously administered. NO was measured in the supernatant by the Greiss reaction. Results are presented as a percentage of the NO levels induced by IL-1 from 6 individual experiments. Hyaluronan of 500-730 kDa, at concentrations higher than 100 µg/ml, caused a significant reduction of NO levels compared with 6,000 kDa HA($p < 0.05$).

ings are in accordance with other studies reporting that high and low MW HA may exhibit different biological effects on other cells and in tissues other than cartilage (2, 5, 11, 17, 18).

HA has been used in OA therapy for several years (3,10). Characterization of the different commercial preparations of HA revealed a marked difference in MW. For this reason we investigated whether the HA size may be an important contributor to bioactivity with respect to inducing levels of NO, prostaglandins and chondrocyte apoptosis. We chose two commercial preparations of HA: a high MW crosslinked hyaluronan (hylan G-F 20; MW 6,000 kDa) and a non-crosslinked HA with a MW of 500-730 kDa. Our results showed that neither HA induced NO or PGE2 synthesis, nor chondrocyte apoptosis. However, these HA preparations have different biological activities. The HA of 500-730 kDa, but not of 6,000 kDa, partially decreased the effect of IL-1 on NO and PGE2 synthesis. Furthermore, both HA preparations were able to reduce the apoptosis induced by a donor of NO such as SNP.

Our results are in accordance with studies using large animals with OA, which showed that HA preparations with MWs within the range of 500-1,000 kDa were generally more effective in reducing the indices of synovial inflammation and restoring the rheological properties of SF than HA preparations with MW > 2,300 kDa (24). However, clinical studies of the efficacy of different weight hyaluronan preparations are not easily comparable; the heterogeneity of these studies limits the drawing of definitive conclusions. For example, some authors have reported that intra-articular sodium hyaluronate was an effective and safe treatment for pain in patients with moderate to severe OAof the knee (25). On the other hand, some studies have concluded that compared with lower molecular weight hyaluronic acid, the highest molecular weight hyaluronic acid may be more efficacious in treating knee OA(26).

The exact mechanism accounting for the efficacy of HA has not been fully elucidated. Some studies have shown a direct effect of hylan, the 6000 kDa HA, on the release of mediators and on nociceptor firing rates (27). Recent publications report that 500-730 kDa HA decreases anti-Fas-induced apoptosis in OAchondrocytes (15) and also reduced apoptosis in an animal model (16). This *in vitro* study also shows that the compound exerts an important effect on chondrocyte apoptosis induced by NO, and on NO and PGE2 synthesis. Some of the biological activities of HAmay be mediated by linking its specific receptors (CD44, CD54 or RHAMM) (4).

In our study we cannot exclude the presence of other mechanisms that block chondrocyte apoptosis such as the trapping of NO molecules. We cannot definitively explain why two commercial HA preparations differed in their effects on PGE2 and NO synthesis, but their different MWs and their different capacity to bind to superficial membrane receptors are possible explanations. We cannot exclude the possibility that structural differences between various preparations of HA, including differences in their secondary or tertiary structure and the extent of crosslinking and rheologic properties may contribute to the differences in their biological activity.

In summary, we provide evidence that HAnot only is a viscoelastic substance, but also may induce important biologic

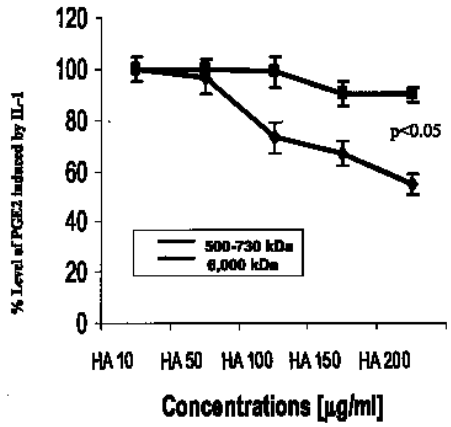

Fig. 2. Effect of two HA preparations on the IL-1 induced synthesis of PGE2. OA cells (75,000 cells/well in a 96-well plate) were incubated with IL-1 at 5 ng/ml for 24 hours. Different concentrations (10-200 µg/ml) of two HApreparations (500-730 kDa and 6000 kDa) were simultaneously administered. The PGE2 content was measured by an enzyme immunoassay. Results are presented as a percentage of the PGE2 levels induced by IL-1 from 6 individual experiments. Hyaluronan of 500-730 kDa, at concentrations higher than 100 µg/ml, caused a significant reduction of PGE2 levels compared with 6,000 kDa HA($p < 0.05$).

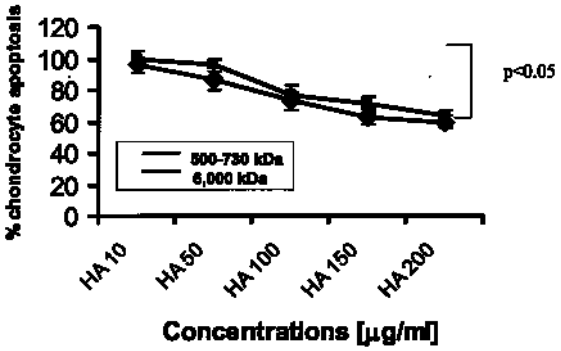

Fig. 3. Effect of two HApreparations on apoptosis induced by sodium nitroprusside (SNP). OAcells were simultaneously incubated with SNPat 2 mM and different concentrations (10-200 µg/ml) of two HApreparations (500-730 kDa and 6000 kDa) for 24 hours. Cells were fixed in 70% ethanol at 4ºC for 60 minutes and stained with propidium iodide (PI, 100 µg/ml). Apoptosis was quantified by flow cytometry on a FACScan. Results are presented as a percentage of the apoptotic cells induced by SNP from 6 individual experiments: % apoptotic cells induced by SNP+ HA/ % apoptotic cells induced by SNPx 100. Both HApreparations reduced the apoptosis in a dose dependent manner. This effect was significant respect to the percentage of apoptosis induced by SNP($p<0.05$).

effects. HA preparations have been grouped as a class; however, this study suggests that HA preparations are different, due to differences in their biologic activity resulting from MW. Whether these results observed *in vitro* can be extrapolated to the clinical setting must be confirmed in future studies.

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