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 HA preparation 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-acetylgalactosamine. It belongs to the glycosaminoglycan family (1). Synoviocytes, fibroblasts and chondrocytes all synthesize HA, which is present in the synovial fluid and the extra-cellular matrix of cartilage (2, 3). HA is 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 HA in 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-deriv- ed 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 triturated using a scalpel. The cartilage pieces were incubated at 37°C 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 37°C for 12 hours. Human chondrocytes were recovered by centrifugation and counted using a Neubauer hemocytometer. The isolated chondro-
cytes (4 x 10^6 cells) were cultured in 
162 cm² flasks (Costar, Cambridge, 
MA) in DMEM medium (Life Technolo-
gies, Paisley, Scotland, UK) supple-
mented with 100 U/ml penicillin, 100 
µg/ml streptomycin, 1% glutamine and 
10% fetal bovine serum (FBS) (Life 
Technologies). Chondrocytes were 
incubated at 37°C in a humidified gas 
mixture containing 5% CO₂ balanced 
with air. In order to ensure the chondro-
cytic phenotype, only high density pri-
mary passage cells were used. Cell via-
bility was assessed by trypan blue dye 
exclusion; stained cells were discarded 
before carrying out experiments.

General experimental procedures 
Chondrocytes were cultured, as de-
scribed above (19), in DMEM medium 
supplemented with 100 U/ml peni-
cillin, 100 µg/ml streptomycin, 1% glu-
tamine and 5% FBS with the addition of 
IL-1 (5ng/ml) and/or HA (10-200 
µg/ml). Two HA preparations with dif-
cerent MWs were used: Hyalgan® (6,000-
kDa, Biomatrix Inc), at 100-200 
µg/ml; 1000,000 cells/well in 6-well plates) were incubated with SNP 
at 2 mM to the two HA prepara-
tions at different concentrations for 24 
hours. Then, cells were fixed in 70% 
ethanol at 4°C for 60 minutes, washed 
and incubated with RNAs (50 µg/ml) 
and propidium iodide (PI, 100 µg/ml) 
for 15 minutes at room temperature in 
the dark, and kept at 4°C. The resulting 
PI fluorescence of the nuclei was mea-
sured 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 apoptot-
ic (hypodiploid) nuclei.

In each experiment, both HA prepara-
tions were tested using cells in tripli-
cate cultures from the same donor; cells 
different donors were never pool-
ed. Cells from the same donor were em-
ployed to carry out a single experiment 
to test the effects of both HA prepara-
tions on NO or PGE2 synthesis or apop-
tosis. Six single assays were performed 
for each parameter studied.

Hyaluronan preparations employed 
Hyalgan® is a highly purified preserv-
ative-free HA in phosphate buffered 
saline (PBS; 10 mg/ml) obtained from 
rooster comb and having a MW of 
500-730 kDa. This HA, prepared follow-
ing the rules approved by the European 
Pharmacopoeia, was kindly supplied 
by Bioiberica S.A. (Spain). The other 
HA used was Synvise® (hylan G-F 20), 
a highly purified formulation of rooster 
comb HA. It is a high MW (6,000 kDa) 
crosslinked HA marketed by Biomatrix 
Inc. in Spain. Solutions of both HApre-
parations 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 ex-
periments. 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 ± 100). 
Results of the effect of HA on apopo-
tosis were calculated as follows: % apop-
tosis induced by SNP+ HA/ % apop-
tosis induced by SNP x 100. Statistical 
analyses were performed with the un-
paired two-tailed Student’s t-test.

Results 
Effect of HA preparations on inflam-
matory 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 HA and 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 ob-
tained when PGE2 synthesis was stud-
ied. Neither HA modified the basal 
synthesis of PGE2 (Table I). However, 
the 500-730 kDa HA decreased the 
levels of PGE2 induced by IL-1 in a dose-
dependent manner (Fig. 2).

Effect of HA preparations on chondro-
cyte apoptosis induced by nitric oxide 
Based on our previous findings, SNPat 
2 mM was employed to induce chon-
drocyte 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 pre-
parations 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 HA decreas-
ed apoptosis by 40% (Fig. 3). This ef-
fct 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.

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>NO (Mean ± SD) (µmoles nitrates)</th>
<th>PGE2 (Mean ± SD) (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>2.9 ± 1.1</td>
<td>270 ± 50.1</td>
</tr>
<tr>
<td>IL-1 (5 ng/ml)</td>
<td>124 ± 20.5</td>
<td>721 ± 94.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MW 570-730 kDa</th>
<th>MW 6000 kDa</th>
<th>MW 570-730 kDa</th>
<th>MW 6000 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA10 µg/ml</td>
<td>2.8 ± 1.2</td>
<td>3 ± 1.3</td>
<td>260.5 ± 41.2</td>
</tr>
<tr>
<td>HA50 µg/ml</td>
<td>3.0 ± 1.6</td>
<td>2.7 ± 1.5</td>
<td>283.1 ± 43.5</td>
</tr>
<tr>
<td>HA100 µg/ml</td>
<td>3.3 ± 1.4</td>
<td>3 ± 1.3</td>
<td>277.3 ± 39.9</td>
</tr>
<tr>
<td>HA150 µg/ml</td>
<td>3.4 ± 1.5</td>
<td>3.4 ± 1.4</td>
<td>281.2 ± 40.1</td>
</tr>
<tr>
<td>HA200 µg/ml</td>
<td>3.6 ± 1.9</td>
<td>3.6 ± 1.7</td>
<td>285.1 ± 45.6</td>
</tr>
</tbody>
</table>

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

Fig. 1. Effect of two HA preparations on IL-1 induced synthesis of NO. OA cells 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 HA preparations (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 6000 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 6000 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 OA of 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 OA chondrocytes (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 HA may 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 HA not only is a viscoelastic substance, but also may induce important biological actions 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).
Effects. HA preparations have been grouped as a class; however, this study suggests that HA preparations are different, due to differences in their biological 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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