The effect of hyaluronan on CD44-mediated survival of normal and hydroxyl radical-damaged chondrocytes

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Summary

Objective: To identify the CD44-receptor-mediated effects of 5–7×10^5 MW hyaluronan (HA, Hyalgan®) on cell viability in normal and damaged human chondrocyte primary cultures isolated from articular cartilage.

Design: Primary cultures of human chondrocytes were established from normal articular biopsies and expanded to the second culture passage. The dose–response effects of HA on the viability of normal cultures were identified. Chondrocytes were then treated with either hypoxanthine (2 mM) and xanthine oxidase (20–60 mU), or with activated polymorphonuclear leukocytes (PMNs) to induce injury. Damaged and control cells were then treated with 5–7×10^5 HA in the previously identified optimal dose of 0.05 mg/ml. Viability was assessed at specific time periods for the chemically and PMN-damaged cells. To identify if HA effects were mediated by the CD44 receptor, chondrocytes were incubated with anti-CD44 antibody at saturating concentrations (5 µg/ml for 100 000 cells) to produce a maximum inhibition of HA binding. Cells were evaluated using the MTT viability assay, histology, electron microscopy and immunohistochemistry.

Results: Direct addition of HA (optimal dose, 0.5 mg/ml) significantly increased cell survival in normal chondrocyte primary cultures (P<0.05). Similarly, addition of this same dose of HA to cultures of free radical-damaged chondrocytes, restored the viability to baseline conditions. Cell viability rates dropped significantly (P<0.05) when CD44 receptor binding was inhibited, indicating that cell growth was mediated by the CD44 receptor.

Conclusions: HA (0.5 mg/ml of 5–7×10^5) significantly increased the viability of normal human chondrocytes in primary culture and restored cell viability to near normal levels after oxidative cell injury.

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Key words: Hyaluronan, Chondrocytes, CD44.

Introduction

Native hyaluronan (HA) is a large glycosaminoglycan that comprises repeating disaccharide subunits of N-acetylglucosamine and D-glucuronic acid. It plays a key role in the structure and organization of the extracellular matrix (ECM)1–3, and can be found in most organs and tissues. A high molecular weight HA molecule is found in abundance in joint synovial fluid. Its unique rheological properties render it highly viscous and of great lubricating capacity: both essential requirements for the integrity of articular cartilage and the overall mechanical performance of synovial joints. In diseases of chronic articular inflammation, the concentration and average molecular weight (MW) of HA have been shown to decrease significantly, resulting in decreased synovial fluid (SF) viscosity4.
and, possibly, in cartilage disruption\textsuperscript{5,6}. These small HA fragments are unable to bind to CD44, the principal HA receptor on chondrocytes\textsuperscript{7,8}. This receptor molecule, a type I transmembrane protein and a member of the cartilage link protein family, is expressed on the surface of chondrocytes\textsuperscript{9,10}, and its binding to HA is essential to both the adhesive and cell-signaling functions of the ECM\textsuperscript{7,11–16}. Chondrocytes are known to highly express CD44.

Fig. 2. Immunolocalization of CD44 on chondrocyte cell surface at 7 days of culture. The presence of CD44 was determined immunohistochemically using a monoclonal antibody against CD44. Cells were counterstained with hematoxylin. (a) Cells show a positive staining for the molecule (red color) (magnification, 400×); (b) negative control.
receptors, which after their binding to HA, result in increased cell proliferation. This effect has been shown to be independent of both transforming growth factor (TGF) and c-myc mRNA, two supposedly critical mediators of cartilage metabolism.

Some studies demonstrated that PMN-generated reactive oxygen radical species (ROS) may be responsible for the HA fragmentation and ECM disassembly in chronically inflamed cartilage of osteoarthritic (OA) patients. Apoptosis has also been recently correlated to the degenerative processes of osteoarthritis. The pharmacological basis of therapy in osteoarthritis should aim to reduce the damage caused by oxygen-free radicals and to stimulate cartilage tissue repair. Intra-articular injection of exogenous HA preparations (Hyalgan®), which are known to have a maximum inhibition of HA binding. Anti-C44 antibodies also inhibited normal baseline proliferation of control cell cultures at 9 days. Each column depicts the mean value of three experiments. Values are expressed as optical density (OD) ± standard deviation (SD). Statistical analyses: Mann–Whitney U test.

Fig. 3. Inhibition of HA binding to chondrocyte CD44 receptor. The addition of monoclonal anti-C44 antibodies to chondrocyte culture medium in the presence of 0.5 mg/ml HA produced a maximum inhibition of HA binding. Anti-C44 antibodies also inhibited normal baseline proliferation of control cell cultures at 9 days. Each column depicts the mean value of three experiments. Values are expressed as optical density (OD) ± standard deviation (SD). Statistical analyses: Mann–Whitney U test.

Materials and methods

HYALURONAN

HA preparations were extracted and purified from rooster comb tissue and had a MW of 5–7×10⁵ Da (Hyalgan®; FIDIA Research Laboratories, Abano Terme, Italy). Four concentrations were evaluated for the effect on the viability of normal chondrocytes in primary culture: 0.25, 0.50, 0.75, and 1.0 mg/ml. The most effective of these doses was then chosen for the subsequent study in damaged cells.

HUMAN CHONDROCYTES

Primary cultures of human chondrocytes were established from articular biopsies taken from normal, non-weight bearing articular cartilage during surgery. Briefly, finely minced and pooled biopsies were treated with serial enzymatic digestion: trypsin 0.25% EDTA 0.02% (Gibco), testicular hyaluronidase (Sigma) and collagenase type I (Sigma). The digested material was then resuspended in complete Ham’s F12 medium supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% L-glutamine, 50 µg/ml l-ascorbic acid (Sigma), 1 ng/ml TGF β1 (Calbiochem), 1 ng/ml insulin (Sigma), 1 ng/ml epidermal growth factor (EGF, Sigma) and 10 ng/ml b-fibroblast growth factor (bFGF, Calbiochem).

Chondrocytes were expanded for 7 days (two passages). Medium was changed every other day and the viability of each cell preparation was tested with trypan blue exclusion. At confluence, cells were trypsinized, detached from the plastic dishes and split at a ratio 1:3.

SUPEROXIDE-GENERATING SYSTEM AND CELL DAMAGE

Two superoxide-generating systems were used to induce cell damage:

(a) Cell monolayers were incubated at 37°C for 30 min with hypoxanthine (2 mM) and xanthine oxidase (20–60 µU) in the presence of FeSO₄ and EDTA (0.2 mM).

(b) Polymorphonuclear leukocytes (PMNs) were isolated from normal human blood by dextran sedimentation followed by Ficoll–Hypaque gradient and hypotonic lysis of erythrocytes. They were then activated by reaction with formyl–methionyl–leucyl–phenylalanine peptide (FMLP) and cytochalasin B. PMNs were seeded on 0.4 µm pore filters that were positioned inside wells containing cultured chondrocytes. In this way, the two cells were grown separately, yet within the same culture environment, communicating and interacting via their metabolic products.

Cell viability was established by the MTT test (see subsequent discussion) and by histological examination of cells.

CELL VIABILITY IN THE PRESENCE OF HA

To evaluate the effects of HA on normal cell viability, human chondrocytes in standard primary culture conditions were grown with varying concentrations (0, 0.25, 0.5, 0.75, and 1 mg/ml) of HA (Hyalgan®) in complete Ham’s F12 medium. At various time points (Days 3, 6, 9), samples were evaluated for viability.

To evaluate the effects of HA on the viability of damaged cells, chondrocytes were cultured in complete Ham’s F12...
Fig. 4. Effect of HA on morphology of cells treated with oxygen-free radicals. Human chondrocytes exposed to oxygen radical flux generated by xanthine oxidase for 30 min: (a) normal human chondrocytes; (b) human chondrocytes exposed to oxygen radical flux with no HA treatment; and (c) with HA treatment (0.5 mg/ml). In (b) and (c), chondrocytes demonstrated cytoplasmic vacuolization induced by free-radical damage, whereas nuclear condensation was observed primarily in untreated samples (panel b). Toluidine Blue staining. Original magnification: 400×.
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medium supplemented as previously described, and were:

(a) treated with HA (0.5 mg/ml), both at the moment and immediately after oxidative damage (chemically or PMN-activated induced) and in all post-injury media changes. Chemically damaged cells were tested for viability after 3, 6 and 9 days, and PMN-damaged cells, after 5 and 10 days.

(b) treated with HA only immediately after chemical oxidative damage. Viability of cell were tested at 3–6–9 days.

Each experiment was performed three times with different cartilage samples taken from different subjects.

HA RECEPTOR BINDING INHIBITION

The mediation of HA effects by the CD44 receptor was tested by receptor-binding inhibition. Human chondrocytes were incubated with a primary mouse anti-human CD44 monoclonal antibody (AnCell) at saturating concentrations (5 µg/ml for 100 000 cells) to produce a maximum inhibition of HA binding.

CELL VIABILITY ASSAY

Viability rates were determined using the MTT-based (Thiazolyl blue) cytotoxicity test according to the method of Denizot and Lang with minor modifications. This test is based on mitochondria viability: only functional mitochondria can oxidize an MTT solution, giving a typical blue-violet end product. Briefly, supernatant was harvested from culture dishes, 1 ml of MTT solution (0.8 mg/ml) was added and samples were returned to the incubator (37°C). After 3 h, supernatant was harvested, 1 ml of extraction solution (10% DMSO in isopropanol) was added, and the samples were read at 540 nm, with 630–660 nm used as the reference wavelength. A calibration curve was established.

ELECTRON MICROSCOPY

Cultured chondrocytes were rapidly washed with fresh medium serum-free at 37°C, rinsed with PBS, pH 7.2, at room temperature and fixed with 2.5% glutaraldehyde in 0.1 M Na–Cacodilate buffer, pH 7.4, for 24 h at 4°C. After washing three times (10 min each) with cold buffer, cells were post-fixed for 20 min at 4°C in 1% OsO₄, and rinsed again with cacodilate buffer. Chondrocytes were then separated with a rubber and dehydrated in a series of ascending water–ethanol mixtures (30–100%) prior to embedding in Epon 812 (Fluka) and polymerizing according to standard techniques. Thin sections were cut with a Reichert–Jung Ultratcut ultramicrotome, collected on copper grids, stained with 2.5% alcoholic uranyl acetate and Reynold’s lead citrate, and finally examined in a CM12
transmission electron microscope (Philips) at an accelerating voltage of 60 kV. Additional sections were also stained with Toluidine blue and examined with light microscopy.

**IMMUNOHISTOCHEMISTRY**

Human chondrocytes were grown on glass coverslips, fixed in acetone for 5 min and incubated with mouse anti-human CD44 antibody in Tris–buffer saline (TBS: 10 mM Tris, 150 mM NaCl, pH 7.4). Coverslips were subsequently treated with a secondary rabbit anti-mouse immunoglobulin (Dako) and then with APAAP (Dako). Lastly, coverslips were stained with Fast red (Sigma) and counterstained with hematoxylin. Positive controls were obtained using lymphocytes and negative controls, by using an irrelevant mouse monoclonal primary antibody.

**STATISTICAL ANALYSIS**

Group data were expressed as mean±standard error. Differences between groups were analyzed using the Kruskal–Wallis test for viability data in the presence of Hyalgan. This test for multiple comparisons was followed by the Mann–Whitney U-test for comparison between groups. Probability values of $P<0.05$ were considered as significant. Data analysis was performed using the Stat View SE Graphics Program (Abacus Concept Inc., Berkely, CA, U.S.A.).

**Results**

**HA AND CELL VIABILITY**

Direct addition of HA was shown to increase normal chondrocyte growth. As shown in Fig. 1, HA induced a dose-dependent pro-proliferative effect up to 0.75 mg/ml, with the greatest efficacy observed at 0.5 mg/ml. Statistical analysis showed that growth enhancement was significantly higher at the 0.5 mg/ml concentration than in cell cultures without HA or with HA at higher (1 mg/ml) and lower (0.25 mg/ml) concentrations (Kruskal–Wallis test for multiple comparisons, $P<0.0205$; and Mann–Whitney U test for comparison between groups, $P<0.05$). At the highest concentration (1 mg/ml), HA demonstrated an opposite effect, decreasing cell viability.

**HA AND CD44 RECEPTOR**

As shown in Fig. 2, immunohistochemical analysis demonstrated that isolated chondrocytes used in the present study highly expressed CD44 receptors. To verify whether the mechanism of HA-enhanced cell proliferation was mediated by the CD44 receptor, binding inhibition experiments were performed using a specific anti-CD44 antibody. In the presence of maximum competitive binding of the CD44 receptor, HA had no effect on cell viability (Fig. 3). This inhibition was statistically significant at 9 days of culture (Mann–Whitney U test for comparison between groups, $P<0.05$). Furthermore, in the absence of exogenous HA, addition of anti-CD44 antibodies to control cell cultures at 9 days slightly, but significantly inhibited their normal baseline proliferation (Mann–Whitney U test for comparison between groups, $P<0.05$).

**HA AND FREE-RADICAL DAMAGE**

Light microscopy demonstrated xanthine oxidase- or PMN-treated cells to have enhanced cytoplasmic vacuolization and chromatin condensation, phenomena that are known to be associated with oxidative damage (Fig. 4). However, nuclear condensation of damaged chondrocytes was more evident in the absence (panel b) than in the presence (panel c) of HA. Ultrastructurally, damaged chondrocytes appeared extensively altered, with marked chromatin condensation and nuclear pyknosis. However, HA-treated cells demonstrated a notable euchromatic nuclear pattern (Fig. 5), even though cytoplasmic vacuolization was present.

MTT viability tests showed that when HA was added to cultures at the moment of chemical [Fig. 6(a)] or activated PMN damage and at every change of media, cell viability was enhanced. This effect was more significant when cell cultures were maintained for 9–10 days [Fig. 6(a),(b)]. Cells chemically damaged and treated with HA only after cell injury, and subsequently at every change of media, also showed enhanced chondrocyte viability (Fig. 7).

**Discussion**

This study explored the effects of HA on the viability of normal and injured human chondrocytes. This primary culture model has been standardized and proven to be physiologically relevant, maintaining the phenotype of cells over time. RT–PCR testing (data not shown) demonstrated that these primary cultures of chondrocytes at second passage expressed collagen type II (80%) and collagen type I (20%), findings that verify the preservation of the chondrocyte phenotype.

Results of this study demonstrated that $5–7\times10^6$ MW HA-enhanced in vitro chondrocyte viability and attenuated the oxidative injury and subsequent decreased survival of these cells in primary culture. The 0.5 mg/ml concentration was identified from the initial dose–response analysis to significantly increase viability of cells in standard culture conditions, whereas the 0.25 and 0.75 mg/ml doses only slightly increased cell survival. Conversely, at 1 mg/ml, an inhibitory effect of HA on the viability of cells in culture was observed. This is in disagreement with the results of Ishida et al., who found that HA was still stimulatory at the 1 mg/ml concentration. This discrepancy is probably due to the different cell populations studied: in the present article, human normal articular cartilage-derived chondrocytes were used, while in the Ishida study, chondrosarcoma-derived chondrocyte-like cells were used.

This study also showed that the effect of HA on cell survival appeared to be mediated by the CD44 receptor. This receptor activity was also shown in samples in which no HA was added, demonstrating that the CD44 receptor also mediates endogenous effects of HAs. These findings are in agreement with several other recent studies, in which the CD44 receptor was shown to mediate the effects of HAs on cell proliferation and motility in normal and tumor cells.

Despite the confusion that still exists with respect to the physiological role of HA and its receptors, and the influence of MW on its pharmacological activity, it is clear that HA, after binding to the cell surface, triggers a cascade of cellular events, such as growth, differentiation, migration, tumor progression and apoptosis. HA also appears to have an important role in scavenging oxygen.
Human chondrocytes exposed to oxygen radical flux underwent morphological and structural changes characterized by cytoplasmic vacuolization and chromatin condensation. In the present study, the morphological changes caused by oxidative injury were less pronounced when cells were treated with HA at the moment of oxidative damage, particularly with regard to the nuclear chromatin structure. More importantly, HA treatment restored the viability of cells damaged with xanthine oxidase or PMN exposure. HA also protected to a similar extent against both chemically generated and activated neutrophil-generated oxidative injury. Activated neutrophils, in fact, are able to release a number of oxidative agents, all potentially involved in the induction of cellular damage.

The present findings demonstrated that one protective function of HA may be enhancement of cell survival. HA not only increased viability of normal chondrocytes, but also restored survival of cells damaged with hydroxyl radicals. In fact, when HA was added to oxidative-stress damaged cells, it restored cell viability to near baseline values, demonstrating indirectly that HA stimulated cellular proliferation. Moreover, HA exerted its beneficial effect both when added to damaged cells at the moment and immediately after the oxidative damage (Fig. 6), and when added only after injury (Fig. 7). The effect of HA may thus be due to both a role in scavenging oxygen radicals, and an enhancement of cell proliferation.

While the MTT test used in the present study may be considered only an indirect measurement of cell proliferation, Beales found a good relationship between viability measured by MTT and cell proliferation measured by [H3] thymidine incorporation. Other researchers reached the same conclusions comparing the BrdU assay and MTT.

In conclusion, these results demonstrated that the tested HA preparation (Hyalgan) was able to enhance human chondrocyte survival/proliferation after exposure to oxygen-free radicals, and that this effect was mediated by the CD44/HA pathway. This enhanced chondrocyte proliferation and survival under conditions of oxidative injury may be one possible therapeutic mechanism of HA in osteoarthritis.

References