Abstract. Objective and design: To determine whether the ability of high molecular weight hyaluronan (HA) to reverse cartilage damage caused by specific catabolic mediators of cartilage damage, fibronectin fragments (Fn-fs), occurs through a low grade of enhanced catabolic events such as enhanced matrix metalloproteinase (MMP) expression or cytokine activities.

Material: HA from 6.8-kDa to 2 million daltons was studied.

Treatment: The ability of HA to enhance matrix metalloproteinase-3 (MMP-3) epitopes and cartilage proteoglycan (PG) degradation neoepitopes was tested in bovine cartilage, as well as the ability of recombinant human interleukin-1 receptor antagonist protein (rhIRAP) to reverse PG depletion in cartilage first exposed to Fn-f.

Results: All HA forms enhanced MMP-3 epitopes and PG degradation in normal undamaged cartilage and in the case of HA800, the degradation was not sufficient to decrease steady state levels of cartilage PG. When HA800 was added to Fn-f damaged cartilage, restoration of PG occurred, but this was blocked by rhIRAP.

Conclusions: These results collectively suggest that some of the repair activity of HA800 is through proteolytic activity which is not sufficient to decrease matrix PG content, but is nonetheless elevated above levels in cartilage not treated with HA800.

Key words: Hyaluronic acid – Hyaluronan – Fibronectin fragment – Cartilage – Cartilage damage – Osteoarthritis – Proteoglycan

Abbreviations: The abbreviations used are: HA, hyaluronic acid; hyaluronan; OA, osteoarthritis; PG, proteoglycan; MMP, matrix metalloproteinase; MMP-3, stromelysin-1; Fn, fibronectin; Fn-f, fibronectin fragment; IL-1, interleukin-1; ECL, enhanced chemiluminescent; HRP, horse radish peroxidase; IGF-1, insulin-like growth factor; recombinant human IGFBP, insulin like growth factor binding protein; rhIRAP, recombinant human interleukin receptor antagonist protein; DMEM, Dulbecco’s modified Eagle’s medium; S.D., standard deviation; DMB, dimethylmethylene blue.

Introduction

Hyaluronic acid or hyaluronan (HA), has been tested in terms of pain alleviation with variable outcomes in the treatment of osteoarthritis (OA) [1–11]. The limitations, difficulties and complexities of some of these studies have been reviewed [12]. In animal models of OA where the structural features of the cartilage tissue can be studied, HA has been shown to slow cartilage degeneration in an anterior cruciate ligament transection model [13, 14], a bilateral osteoarthritis animal model [15] and a partial meniscectomy rabbit model of OA [16, 17]. Little is known of how HA might express this activity. In a study of a rabbit anterior cruciate ligament transection model, HA reduced cartilage damage but did not affect cartilage matrix metalloproteinase (MMP) production [18]. HA also suppressed nitric oxide production in the meniscus and synovium in a rabbit OA model [19], but did not suppress NO synthesis in vitro in experimentally induced OA cartilage [20].

In terms of effects of HA on cytokine activities, while HA has been shown to be ineffective in controlling cytokine expression in OA chondrocytes and synoviocytes [21], HA has been shown to reverse cytokine activities in normal cartilage [22–24] and specifically, to inhibit IL-1 induced superoxide anion in bovine chondrocytes [25] and IL-1 mediated PG synthesis suppression [26].

In order to investigate the mechanism by which HA suppresses cartilage damage, we have utilized a model of cartilage damage based on the ability of fibronectin fragments...
Materials and methods

All common chemicals and reagents as well as an endotoxin assay kit were from Sigma Chemical Co. (St. Louis, MO). HA forms of 6.8, 20, 60, 250 and 800-kDa and 5-aminofluorescein 800-kDa HA were provided by Seikagaku Corporation (Tokyo, Japan). [35S] sulfate (43 Ci/mg S) was from ICN Biomedicals Inc. (Costa Mesa, CA). Monoclonal antibody to human MMP-3 and a Unitekt kit for immunochemical detection of primary antibody was from Oncogene Science (Cambridge, MA). HRP conjugated rabbit anti-sheep IgG (Sigma # A-5054) was from Sigma Chemical Co. (St. Louis, MO). NITEGE and VDIPEN anti-serum were kindly provided by Dr. D. Visco (Merck Research Laboratories, Rahway, NJ). The Enhanced Chemiluminescent (ECL) kit used for Western blot detection was the Super Signal Chemiluminescent Substrate kit for HRP from Pierce Chemical Co. (Rockford, IL). Detoxi-Gel was also obtained from Pierce Chemical Co. Recombinant human IL-1β (rhIL-1β; cat #: 201-LB) was used to confirm the blocking efficacy of recombinant human interleukin-1 receptor antagonist protein (rhIRAP). IL-1β and rhIRAP (cat # 280-RA) were from R&D Systems Incorporated (Minneapolis, MN).

Isolation of the Fn-f

Fn-fs were generated from human fibronectin (Fn) as described [41]. The Fn-f tested was a well-characterized amino-terminal 29-kDa Fn-f that has been found to be the most potent [41] and is referred to as the Fn-f. The Fn-f solution was treated with detoxi-Gel prior to use and was sterile filtered. Assays of endotoxin with a kit from Sigma Chemical Co. showed levels <50 pg/mg of protein.

HA forms

The HA forms are denoted throughout in terms of kDa, i.e. HA of 800-kDa is HA800. HA of 2 million was denoted HA2mil. All HA forms were treated with detoxi-gel to remove trace endotoxin prior to use. Assays of endotoxin using a kit from Sigma Chemical Co. showed levels <50 pg/mg HA. Tests of activities of 50 pg/mg of LPS in PG degradation and PG synthesis assays showed insignificant activity. As separate additional controls all HA solutions were adjusted to 10 µg/ml polymixin B sulfate. The additions were found not to significantly alter HA activities. HA solutions did not routinely contain polymixin B sulfate.

Explant cultures

Culturing of slices of bovine metacarpophalangeal cartilage from 18 month old bovines was performed as described [41–45] in DMEM containing 50 U/ml penicillin-streptomycin in 10% serum/DMEM or in DMEM alone and with 50 to 80 mg cartilage per 1.5 ml/well. The cartilage was allowed to equilibrate for two days in 10% serum/DMEM or DMEM alone, the conditions to be used for the experiment, before experiments were begun. The media were changed every other day for 10% serum/DMEM cultures but were not changed for kinetic assays of PG degradation in serum-free cultures.

Assays of kinetics of PG degradation

Kinetic assays were performed as described [42]. Briefly, cultures were performed in serum-free DMEM as well as in 10% serum/DMEM and the media were not changed during the culture. On each of days 1–6, 50 ml aliquots of the culture media were removed and assayed. Although this resulted in a decrease in culture well volume from 2.0 to 1.70 ml or 15%, the final calculations of PG content were related to weight of cartilage and so the volume change was not corrected. The PG content was converted to mg PG/mg cartilage for each of three similar wells and the amounts graphed vs day in culture to determine rate constants of mg PG/mg cartilage/day by linear regression. The PG content of the slices was also measured at the end of the experiment after subjecting cartilage to papain digestion as described [34].

Assays of effect of HA800 or rhIRAP on PG content of cartilage in 10% serum cultures

In order to test whether the reparative activity of HA800 involved cytokines, bovine cartilage was cultured in 10% serum/DMEM with 100 nM Fn-f from days 0–7 to deplete PG. A combination of HA800 and rhIRAP was then added after the damage phase at day 7. At day 7, some of the cultures were exposed to 1 mg/ml HA800, while others were exposed to 1 mg/ml HA800 with 1.0 µg/ml rhIRAP for up to 14 days. PG content was analyzed every 7 days. PG contents were determined on papain digests using the DMB assay as described [34]. The PG contents were reported as µg PG/mg wet weight cartilage.