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An *in vivo* cross-linkable hyaluronan gel with inherent anti-inflammatory properties reduces OA cartilage destruction in female mice subjected to cruciate ligament transection

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SUMMARY

Objective: To explore the possibility of cartilage protection in osteoarthritis (OA) by intraarticular injection of a chemically modified hyaluronan (HA) gel and investigate whether the chemical modifications provide intrinsic anti-inflammatory activity.
Method: OA was induced in C57BL/6 mice by anterior cruciate ligament transection (ACLT) and HA gel or carbazate-modified component was injected intra-articularly. Assessment of cartilage rescue was performed by histology, immunohistochemistry and TUNEL analysis. Serum levels of proinflammatory cytokines were evaluated with cytometric bead array, measuring IL-1β, TNF, IFN-γ, KC/CXCL1 and MCP-1.
Results: Intraarticular injection of the HA gel showed significantly reduced cartilage destruction and decreased osteophyte formation. Besides the biological and biomechanical effects of HA, we investigated lipid peroxidation products as an alternative inflammatory and potential mechanism contributing to OA. To address this, injection of the carbazate-modified component alone was performed, which also demonstrated a cartilage-saving effect. Besides the cartilage amelioration effects, decreased apoptosis, 4-hydroxynonenal (4-HNE) and MHC class II staining was recorded. No changes in serum levels of proinflammatory cytokines were detected.
Conclusion: We have shown that the HA gel has an anti-destructive effect on articular cartilage (AC). Our results demonstrated that the carbazate-modified component could suppress apoptotic events, potentially by quenching of ROS/LPO products such as 4-HNE in OA joints. Modification of the HA molecule offers opportunities to introduce (covalent) coupling of additional molecules to the gel, with controlled retention and subsequent release in the joint.

Introduction

Osteoarthritis (OA) is a common disease of aged populations and one of the leading causes of disability. Around 25% of the population above 60 years suffers from OA and associated pain and disability are major contributors to the reduced quality of life experienced by many patients. OA is mainly characterized by destruction of AC caused by uncontrolled breakdown of extracellular matrix (ECM). However, the disease is not restricted to cartilage alone and can be considered as a chronic disease of the whole joint, including AC, meniscus (M), ligament, and periarticular muscle. Classical proinflammatory cytokines such as IL-1β or TNF are known to be upregulated in OA[1,2] and although these molecules are targets for successful therapeutic intervention in rheumatoid arthritis (RA), neutralization of IL-1β or TNF have shown no or modest effects in OA[2].

Besides inflammatory mediators such as TNF and IL-1β, the oxidative milieu associated with the low-grade inflammation in OA joints contributes to destruction[3]. Presence of free radicals and reactive oxygen species (ROS) has long been recognized to directly inflict damage to lipids. The peroxidation of phospholipids results in production of highly reactive aldehydes, such as malondialdehyde and 4-hydroxynonenal (4-HNE)[4]. Similar to ROS, aldehydes

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are electrophiles that could bind to nucleophilic groups in proteins, but with a longer half-life, leading to damage reaching also neighboring cells. Under high lipid peroxidation rates the extent of oxidative damage exceeds the abilities of repair mechanisms. As a result, cells become apoptotic which leads to potential development of various pathological states. In OA, chondrocyte apoptosis has been shown to play a critical role in initiation and progression of the disease[5–7]. Synovial fluid from OA patients contains elevated levels of 4-HNE and ROS and decreased levels of antioxidants, making them interesting targets for OA treatment[3].

In addition to anti-inflammatory treatment prescribed to OA patients today, viscosupplementation, i.e., intraarticular injections of structural and lubricating agents such as hyaluronan (HA), are employed to decrease pain and improve joint function[8,9]. HA injections are a well-tolerated therapy and several endogenous functions of HA support its suitability as a biomaterial or intraarticular drug[10]. However, clinical effects are modest with debated outcomes and endogenous HA has a rapid turnover[11–13]. Thus, HA may serve as a foundation for development of better formulations based on HA, with its documented tolerability and possibility of chemical modification. For instance, application of *in vivo* forming gels of chemically modified HA would not only overcome the rapid clearance but provide the opportunity to encapsulate or bind anti-inflammatory or therapeutic molecules in a slow release formula for local delivery of disease modifying drugs (DMOADs).

In this study, we have explored such a HA gel and investigated if it can reduce the cartilage destruction observed in experimental OA. We established an experimental OA model in mice and evaluated the therapeutic efficacy of the HA gel in suppressing cartilage destruction. The HA gel is based on multifunctional components, which form gels *in situ* upon mixing. Specifically, it consists of an aldehyde-modified HA (HAA) component and a carbazate-modified polyvinyl alcohol (PVAC) component, which react rapidly *in situ*, forming cross-links that result in a gel-like scaffolding structure[14–17]. We also explored whether the aldehyde-reactive component alone could reduce cartilage degradation and potential mechanisms of action.

Methods

Animals

Nine week old female C57BL/6J mice were purchased from Charles River and housed in specific pathogen-free facilities at Karolinska University Hospital. Animals were acclimatized at least 7 days prior to any intervention. All experiments were approved by the Stockholm North Ethical Committee (Dnr N535/11, N215/14). The mice had free access to water and standard rodent chow. A 12-h light/dark cycle was maintained at all times.

HA gel materials

The HA gel consists of two components; HAA and carbazate-modified PVAC, which were prepared according to previously described methods[15,16]. Briefly, HAA was dissolved at a concentration of 15 mg/ml in PBS and filtered through a 0.45 μm sterile filter. PVAC was dissolved at a concentration of 2.5 mg/ml in deionized water and passed through a 0.2 μm sterile filter. Components HAA and PVAC were subsequently loaded into 1 ml sterile luer-lock syringes at a volume ratio of 3:2 to ensure stoichiometric cross-linking of the active groups in each component. The tips of the syringes were connected with a luer-lock adapter and the components were mixed at room temperature for 15 s to allow gelation to start prior to injection into the knee joints.

Experimental OA and treatments

OA was induced by surgically destabilizing the left knee joint through anterior cruciate ligament transection (ACLT). A skin incision was made, and a closed method utilizing a 15 blade scalpel to induce cruciate transection was performed without opening the joint. After transection the skin incision was closed and 10 μl HA gel, PBS or carbazate-modified component was injected into the joint according to treatment schedule. Three intraarticular injections were performed; at time of surgery, after 1 week and after 2 weeks. For the HA gel treatment three independent experiments were performed and the data is displayed together with $n = 13$. For treatments with the carbazate-modified component $n = 5$. The surgery was performed under isoflurane anesthesia and post-operative pain relief was provided by administering buprenorphine (0.3 mg/ml) subcutaneously the first 24 h after surgery. The mice could move around freely in the cages after surgery and were monitored daily. Eight weeks after surgery, the animals were sacrificed and joints processed for histopathological examination. For serum cytokine analysis, blood samples were collected from the lateral saphenous vein (~100 μl) preoperatively, 3 days, 2 weeks postoperatively and then every second week until termination. For histological analysis of OA progression in untreated mice, animals were sacrificed at 2, 6 and 8 weeks.

Histologic assessment

Total knee joints were fixed in 4% phosphate buffered formaldehyde for 24 h, decalcified in EDTA solution before dehydration and then embedded in paraffin. 6 μm thick frontal sections were cut and serial sections collected. For histological scoring representative sections were stained with Safranin-O/Fast green and semi-quantitatively scored according to recommendations for mice from the OARSI histopathology initiative[18]. All four quadrants of the joint were assessed separately and given a score from 0 to 6 according to Table I, the sum was calculated to achieve the total score of the joint. The sections were evaluated by two independent observers blinded to the identity of the specimens.

TUNEL staining

To detect apoptotic cells sections were stained by TUNEL staining (Trevigen, R&D systems) according to the manufacturer's protocol. Paraffin embedded sections were deparaffinized, rehydrated in graded alcohols and immersed in 1 × PBS for 10 min. Sections were covered with 15 μg/ml Proteinase K and incubated for 20 min in room temperature. Endogenous peroxidase was inactivated by 3% H_2O_2 in methanol for 5 min. Sections were immersed in terminal deoxynucleotidyltransferase (TdT) buffer for 5 min and then covered with Labeling Reaction Mix and incubated for 60 min at 37°C in a humidity chamber. The reaction was inactivated by TdT Stop Buffer for 5 min. Samples were covered by Strep-HRP solution and incubated for 10 min at 37°C in a humidity chamber and then immersed in 3,3′-diaminobenzidine (DAB) solution for 7 min. For counterstaining, sections were immersed in 1% Methyl green for 5 min. As a positive control to the method, TACS nuclease was used to induce DNA damage. The number of apoptotic cells compared to total number of cells in the AC was analyzed by two independent observers blinded to the identity of the specimens using a Leica microsystems and Leica QWin V3 tips cell count image analysis program (Leica Microsystems, Wetzlar; Germany).