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Deficiency of hyaluronan synthase 1 (*Has1*) results in chronic joint inflammation and widespread intra-articular fibrosis in a murine model of knee joint cartilage damage

D.D. Chan †, W.F. Xiao ‡, J. Li †, C.A. de la Motte §, J.D. Sandy ‖ ¶, A. Plaas † ‖ *

† *Division of Rheumatology, Department of Internal Medicine, Rush University Medical Center, Chicago, IL, USA*
‡ *Department of Orthopedics, Xiangya Hospital, Central South University, Changsha, Hunan, China*
§ *Department of Pathobiology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA*
‖ *Department of Biochemistry, Rush University Medical Center, Chicago, IL, USA*
¶ *Department of Orthopedic Surgery, Rush University Medical Center, Chicago, IL, USA*

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SUMMARY

Objective: Articular cartilage defects commonly result from traumatic injury and predispose to degenerative joint diseases. To test the hypothesis that aberrant healing responses and chronic inflammation lead to osteoarthritis (OA), we examined spatiotemporal changes in joint tissues after cartilage injury in murine knees. Since intra-articular injection of hyaluronan (HA) can attenuate injury-induced osteoarthritis in wild-type (WT) mice, we investigated a role for HA in the response to cartilage injury in mice lacking HA synthase 1 ($Has1^{-/-}$).

Design: Femoral groove cartilage of WT and $Has1^{-/-}$ mice was debrided to generate a non-bleeding wound. Macroscopic imaging, histology, and gene expression were used to evaluate naïve, sham-operated, and injured joints.

Results: Acute responses (1–2 weeks) in injured joints from WT mice included synovial hyperplasia with HA deposition and joint-wide increases in expression of genes associated with inflammation, fibrosis, and extracellular matrix (ECM) production. By 4 weeks, some resurfacing of damaged cartilage occurred, and early cell responses were normalized. Cartilage damage in $Has1^{-/-}$ mice also induced early responses; however, at 4 weeks, inflammation and fibrosis genes remained elevated with widespread cartilage degeneration and fibrotic scarring in the synovium and joint capsule.

Conclusions: We conclude that the ineffective repair of injured cartilage in $Has1^{-/-}$ joints can be at least partly explained by the markedly enhanced expression of particular genes in pathways linked to ECM turnover, IL-17/IL-6 cytokine signaling, and apoptosis. Notably, *Has1* ablation does not alter gross HA content in the ECM, suggesting that HAS1 has a unique function in the metabolism of inflammatory HA matrices.

Introduction

Traumatic injuries to articular cartilage of the knee can result from excessive surface contact stresses after blunt impact or torsion, which occur frequently during sports and military training[1]. Resulting patellar dislocation[2], joint incongruity, and instability can predispose to osteoarthritis (OA)[3]. Responses to cartilage injury share many features of wound healing, such as innate inflammation[4] and activation of multipotent progenitor cells in the synovium or the articular surface[5]. However, in many cases, the repair response leads to fibrotic remodeling and scarring of the joint lining tissues, subchondral bone sclerosis, and chondrophyte or osteophyte development at the articular margins.

Whereas chronic inflammation is widely recognized as a driving factor in OA, the concept of a pathogenic role for fibrotic scarring is less well-studied[6]. Evidence for focal scarring has been reported for synovial tissue and cartilages from both animal model and human OA[7], each of which exhibit activation of multiple genes associated with collagen production and deposition (*CRLF1*, *PLOD2*, *LOX*,

* Address correspondence and reprint requests to: A. Plaas, 1653 West Congress Parkway, Jelke Building, Suite 1413, Chicago, IL 60612, USA. Tel: 1-312-942-7194; Fax: 1-312-563-2267.
E-mail addresses: deva_chan@rush.edu (D.D. Chan), wenfeng_xiao@163.com (W.F. Xiao), jun_li@rush.edu (J. Li), delamoc@ccf.org (C.A. de la Motte), jsandy44@gmail.com (J.D. Sandy), anna_plaas@rush.edu (A. Plaas).

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COL1A1, *COL5A1*, *TIMP1*). In addition, other laboratories[8–10] also reported high expression of COL1A1, COL2A1, COL3A1, and COL5A1 in human OA cartilages. Moreover, mice deficient in genes that enhance collagen matrix formation and turnover in wound healing (*Adamts5*, *Ddr2*, *Mmp13*, *Sdc4*, and *Tgm2*[11–14]) were variably protected from surgically induced OA.

In dermal wound healing, early inflammation is followed by formation of granulation tissue containing progenitor cells embedded in a provisional extracellular matrix (ECM) of collagens, fibronectin, hyaluronan (HA), and hyalectans[15]. These cells, following re-epithelialization, mature into fibroblasts to generate the functional collagenous repair tissue. Correspondingly, following cartilage injury, proliferation of cells and ECM deposition in synovial lining and adjacent adipose or joint capsule tissues occurs. This response can progress into fibrotic remodeling, often reported in inflammatory arthritis[16]. A similar response also develops in the meniscal destabilization mouse model of OA, where, at 2–4 weeks[17], an inflammatory period is followed by elevated expression of profibrotic mediators such as type III collagen (*Col3a1*), fibromodulin (*Fmod*, a catalyst for TGF-β1 signaling), and prolargin (*Prelp*, an inhibitor of osteoclastic activity).

To examine repair responses specifically in the context of articular cartilage injury, we have adapted a murine model induced by surgical excision of cartilage from the patellar groove[18]. This allowed spatiotemporal macroscopic and microscopic evaluation of whole joint-responses and assay of gene expression in inflammatory, pro-fibrotic and ECM pathways, in both intact joints and separated tissue pools (meniscus and synovium (Men/Syn), cartilage and subchondral bone (C/SCB), and patellar tendon (PT)).

We have also examined such injury responses in mice deficient in HA synthase 1 (*Has1*), which have previously been reported to exhibit an aberrant dermal healing phenotype[19]. We show that Has1$^{-/-}$ mice, although not defective in overall HA production, are not able to control post-injury joint inflammation and develop extensive intra-articular scarring and severe OA-like symptoms.

Methods

Murine cartilage injury model

Wild-type (WT) and Has1$^{-/-}$ male C57Bl/6 mice (10–12 weeks old, ~30 g) were used under approval of the Rush University Institutional Animal Care and Use Committee. Routinely, four C57Bl/6 males were caged with one C57Bl/6 female littermate, to minimize male aggression and prevent wounding in the pre-op and post-op maintenance periods. After anesthesia, an ~8-mm medial para-patellar incision was made on the right knee, medial para-patellar arthrotomy was performed, and the patella laterally luxated. Cartilage was debrided along the distal groove with a #15 scalpel without penetration of the subchondral bone. Joint surfaces were lavaged with sterile saline, and the patella repositioned, before the muscle layer and skin were closed with 6-0 Vicryl sutures. Supporting ligaments, menisci, and other cartilage surfaces were not damaged [Fig. S-1(A)], and no changes were detected in the contralateral joint post-injury [Fig. S-1(B)]. Sham surgery included all steps except cartilage debridement. Mice were maintained at cage activity during the 4-week post-surgery period. The minimal number of mice needed for each outcome was determined based on previous studies[20], and the numbers in each experimental group are given (Table S-1).

Macroscopic joint imaging, histology, and HA staining

Joint-wide pathology was assessed in operated and contralateral joints as previously described[20]. For histology, whole joints (after removal of skin and muscle) were fixed in 10% neutral-buffered formalin, decalcified with 5% EDTA in PBS, paraffin-embedded, and microtome-cut into 6-μm sections across the entire joint[20]. Sections 1–60, 61–120, and 121–190 spanned medial, central groove, and lateral compartments, respectively. Slides 1/2, 22/23, 42/43, 62/63, 82/83, 102/103, 122/123, 142/143 and 182/183 were stained with Safranin O (SafO), and adjacent sections with hematoxylin and eosin (H&E) or biotinylated HA binding protein (bHABP) to localize HA. It should be noted that the histological analysis was not used in this study to generate a numerical scale for cartilage grading (as per OARSI guidelines) but evaluated, in combination with the macro-images, to describe structural alterations in multiple tissue types adjacent to the injury and throughout the whole joint.

Quantitative PCR (qPCR)

For gene expression in whole joints from naïve, sham, and injured groups (n = 3–4, detailed in Table S-1), hind legs were harvested immediately after sacrifice, the skin and muscle removed, and knee joints isolated by sharp dissection through the growth plates, prior to storage at −20°C in RNAlater (Table S-1). To prepare separate tissue pools, twelve joints were used for Men/Syn or PT, and two for C/CSB. RNA purification, cDNA synthesis, and qPCR (3 technical replicates) with Taqman®-primers (Table S-2) was done as previously described[20]. Transcript abundance was calculated as $1000 \times 2^{-\Delta Ct}$, with ΔCt = [Ct(gene of interest) − Ct(*Gapdh*)] and Ct > 35 considered "non-detectable" (ND). RT^2 Profiler PCR Arrays (Qiagen) were used for fibrosis (PAMM-120ZA) and NF-κB signaling target (PAMM-225ZA) genes. Injury-induced fold-change in expression was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ = [ΔCt(post-injury time point) − ΔCt(naïve)]. Gene groupings indicated by Qiagen and Metacore™ software analysis of expression data were used to determine pathway associations.

Data and statistical analysis

For statistical comparisons across time points and between genotypes, qPCR assays were performed on whole joints (each joint an experimental unit), from naïve, sham, and injury groups, because the large number of mice (12 per experimental unit) required for generating multiple pools of separated tissue types was outside the scope of this study.

Gapdh Ct values from WT and Has1$^{-/-}$ samples were pooled to confirm normality of Ct values with the Shapiro-Wilk test, and analysis of variance (ANOVA) was used to compare *Gapdh* Ct values across groups to confirm selection of the housekeeping gene. For all combinations of WT/Has1$^{-/-}$ naïve/sham/injury joints (6 in total), Ct values were confirmed with the Shapiro-Wilk test to be normally distributed. For each gene, ANOVAs were performed on ΔCt values to compare expression in the following subgroups: 1-way ANOVA for WT naïve vs 12 or 28 day post-sham; 2-way ANOVAs for WT vs Has1$^{-/-}$, naïve vs 12 or 28 day post-injury (main effects: experimental end point, genotype). ANOVAs were followed by post hoc analysis of statistically significant effects using unpaired, two-tailed Student's t tests with Bonferroni correction of the P value for multiple comparisons against naïve. Since only two end points at biologically distinct phases of sham and injury response were compared to naïve, Bonferroni correction was chosen as the most conservative option for post hoc comparisons after ANOVA.

For each gene from the arrays, unpaired two-tailed Student's t tests were used to compare between WT and Has1$^{-/-}$ ΔCt values at naïve. Similar analysis was performed to compare array results of WT naïve to WT sham. ANOVA was used to compare genotypes for ΔCt values after injury (to determine the overall effect of genotype),