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Superficial zone chondrocytes in normal and osteoarthritic human articular cartilages synthesize novel truncated forms of inter-alpha-trypsin inhibitor heavy chains which are attached to a chondroitin sulfate proteoglycan other than bikunin

Y. Yoshihara M.D., Ph.D.†‡, A. Plaas Ph.D.†§*, B. Osborn B.S.‖, A. Margulis M.D.†, F. Nelson M.D.¶, M. Stewart B.V.Sc, Ph.D.#, M. S. Rugg Ph.D.††, C. M. Milner Ph.D.‡‡, A. J. Day Ph.D.‡‡, K. Nemoto M.D., Ph.D.‡ and J. D. Sandy Ph.D.†§§

† *Department of Biochemistry, Rush University Medical Center, Chicago, IL, USA*
‡ *Department of Orthopaedic Surgery, National Defense Medical College, Saitama, Japan*
§ *Department of Internal Medicine (Rheumatology), Rush University Medical Center, Chicago, IL, USA*
‖ *Department of Internal Medicine, College of Medicine, University of South Florida, Tampa, FL, USA*
¶ *Bone and Joint Center, Henry Ford Hospital, Detroit, MI, USA*
# *Veterinary Clinical Medicine, University of Illinois at Urbana-Champaign, Urbana, IL, USA*
†† *MRC Immunochemistry Unit, University of Oxford, OX1 3QU, UK*
‡‡ *Faculty of Life Sciences, University of Manchester, M13 9PT, UK*
§§ *Department of Orthopaedic Surgery, Rush University Medical Center, Chicago, IL, USA*

Summary

Objective: We have examined the occurrence of the inflammation-associated inter-alpha-trypsin inhibitor (IαI) components, bikunin, heavy chain (HC)1 and HC2 in normal cartilage and osteoarthritis (OA) cartilage and synovial fluids.

Design/methods: Cartilage extracts from normal donors and late-stage OA patients, and synovial fluids from OA patients were studied by Western blot with multiple antibodies to bikunin, HC1 and HC2. Cell and matrix localization was determined by immunohistochemistry and mRNA by RT-PCR.

Results: Bikunin·chondroitin sulfate (CS) and IαI were abundant in OA cartilages, but virtually undetectable in normal. In both OA and normal cartilages, HCs were largely present in a novel C-terminally truncated 50-kDa form, with most, if not all of these being attached to CS on a proteoglycan other than bikunin. Synovial fluids from OA patients contained bikunin·CS and full-length (~90 kDa) HCs linked to hyaluronan (HA) as HC·HA (SHAP·HA). Immunohistochemistry showed intracellular and cell-associated staining for bikunin and HCs, consistent with their synthesis by superficial zone chondrocytes. PCR on multiple human normal and OA cartilage samples detected transcripts for HC1 and HC2 but not for bikunin. In OA cartilages, immunostaining was predominantly matrix-associated, being most intense in regions with a pannus-like fibrotic overgrowth.

Conclusion: The truncated structure of HCs, their attachment to a proteoglycan other than bikunin, PCR data and intracellular staining are all consistent with synthesis of HC1 and HC2 by human articular chondrocytes. The presence of bikunin·CS and IαI in OA cartilage, but not in normal, appears to be due to diffusional uptake and retention through fibrillated (but not deeply fissured) cartilage surfaces.

Key words: Osteoarthritis, Inflammation, Hyaluronan, Chondroitin sulfate, Proteoglycans, Inter-alpha-trypsin inhibitor, Synovial fluid.

Introduction

Inter-alpha-trypsin inhibitor (IαI) is composed of two heavy chains (HCs), HC1 and HC2 attached by ester linkages to the single chondroitin sulfate (CS) chain on the proteoglycan form of bikunin (otherwise known as bikunin·CS)[1,2]. For many years, functional knowledge of IαI was restricted to inhibition of serine proteinases (e.g., plasmin) by bikunin[3]. However, the occurrence of IαI components in many human tissues[4–7] suggested a wider physiological importance[8]. In this regard, the role of IαI in delivering HCs for the covalent modification of hyaluronan (HA) to form HA·HC (or SHAP·HA) complexes at sites of inflammation[9,10], has received much attention (reviewed in Ref. 11). It is understood that HC·HA complexes are generated *via* two sequential transesterification reactions, where the C-terminal aspartates of HC1 and HC2 are initially attached, *via* ester bonds, to the C-6 hydroxyl groups of *N*-acetylgalactosamine (GalNAc) in the CS chain of bikunin·CS during IαI biosynthesis. Extracellularly, the HCs are transferred from IαI to TSG-6 (to give TSG-6·HC intermediates) and

*Address correspondence and reprint requests to: Dr Anna Plaas, Ph.D., Department of Internal Medicine, Section of Rheumatology, Rush University Medical Center, Professional Building, Suite 1017, 1725 W Harrison Street, Chicago, IL 60612, USA. Tel: 1-312-942-7194; Fax: 1-312-942-3053; E-mail: anna_plaas@rush.edu
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finally from TSG-6 onto *N*-acetylglucosamine (GlcNAc) residues of HA[12,13].

HC·HA has different properties than free HA, which has been attributed to cross-linking *via* non-covalent HC–HC interactions[14]. HC·HA has been implicated in pathological inflammation with large amounts present in the synovial fluid of rheumatoid arthritis patients[14,15]. In this regard, HCs have been shown to enhance the binding of HA to CD44, thereby promoting leukocyte adhesion[16]. Thus HC·HA formation is a key component of matrix remodeling both in pathological contexts, where it has been associated with both pro- and anti-inflammatory effects, and in normal physiology. Furthermore, there is evidence that HCs might form ester linkages to CS on PGs (proteoglycans) in addition to bikunin·CS[17,18], suggesting that HCs play a diverse role in matrix organization.

A role for IαI components in modifying joint disease is suggested by reports on increased concentrations of bikunin, HCs and/or TSG-6–IαI complexes in the serum or synovial fluid of RA (rheumatoid arthritis) and osteoarthritis (OA) patients[14,15,19–21]. IαI is known to be abundant in serum and to be synthesized in the liver, but it has also been detected in a variety of other tissues. Here we set out to answer the following questions: (1) Do chondrocytes of normal human cartilage produce bikunin, HC1 and HC2, and what complexes do these form in cartilage? (2) Are there HC·HA complexes in human cartilage? (3) Is the abundance or structure of IαI components altered in OA? And (4) are the IαI components in OA synovial fluid the same as those found in cartilage? Our novel findings may provide new tools for the evaluation of inflammatory processes and turnover of the HA-associated matrix in human OA.

Methods

Many of the materials and methods used here have been described in detail previously[22].

ANTIBODIES

Anti-peptide antibodies to HC1 (JSCQVQ (also called QVQ), against the sequence 383-QVQESLPELSN-393) and HC2 (JSCEAN (also called EAN) against the sequence 401-EANNLGLLDPN-411) were prepared by Affinity Bioreagents, Golden, CO. These were used at 10 μg/ml for immunohistochemistry and 1 μg/ml for Western blot. The anti-bikunin antiserum (ab43073 (also called 073), from Abcam Inc., Cambridge, MA) was raised against full-length recombinant human protein and was diluted 1:400 for immunohistochemistry and 1:2000 for Western analysis. Anti-peptide antisera were raised in rabbits by Mimotopes Pty Ltd. (Clayton, Australia) against the N-terminal peptides of human HC1 (CP7; SATGRSKSSEC), HC2 (CP10; SLPGESEEMMC) and bikunin (CP6; AVLPQEEEGSC) and the C-terminal peptide of human HC2 (MIM-7; CESTPPPHVMRVE), where the peptides were coupled to a diphtheria toxoid carrier protein *via* non-authentic cysteine residues at their C- or N-termini (see Ref. 23 and Supplementary Fig. 1 for verification of antisera specificity). The antisera were used at 1:5000 for Western blotting and 1:500 for immunohistochemistry.

EXTRACTION OF HUMAN CARTILAGES AND CsCL GRADIENT CENTRIFUGATION

Femoral condylar cartilage was from five individuals with no history of joint disease (age range 28–64 y). The 28 y sample also included tibial plateau cartilage and all were used within 24 h of death. The cartilage surfaces were either grade 0 (28 y, 29 y, 47 y, and 64 y) or grade 1 (63 y), using the grading system described previously[24]. For the grade 1 patient, cartilage was removed only from the non-fibrillated areas. Osteoarthritic cartilage samples were pooled from grades 3 and 4 tibial plateaus from seven patients (age range 50–75 y) undergoing total knee arthroplasty at Henry Ford Hospital under an IRB-approved protocol. Finely sliced tissue was extracted in 4 M guanidine·HCl/0.05 M sodium acetate/0.1 mM AEBSF (2-aminoethyl)-benzenesulfonyl fluoride)/5 mM EDTA/5 mM iodoacetamide/0.5μg/ml pepstatin (pH 6.8) at 4°C for 48 h and, after filtration through glass wool it was run in a dissociative CsCl gradient (starting density 1.5 g/ml) and fractions D1 (about 1.65 g/ml) through D6 (about 1.40 g/ml) were dialyzed against water and dried under vacuum.

SEQUENTIAL EXTRACTION OF HUMAN CARTILAGES

A sequential associative/dissociative extraction protocol was also developed to examine tissue associations for the different species. This involved freeze-milling of the cartilage and extraction (3 ml/g wet wt.) with 50 mM Tris–HCl/100 mM NaCl (pH 7.0) for 2 h at 4°C (Extract 1) followed by 50 mM Tris–HCl/150 mM NaCl/0.5% (w/v) NP-40/0.5% (w/v) deoxycholate (pH 7.0) for 24 h at 4°C (Extract 2) and finally with 4 M guanidine·HCl/0.01% (w/v) CHAPS/Tris (pH 8.0) for 24 h at 4°C (Extract 3). All extractants contained proteinase inhibitors (one tablet of Complete Mini©, Roche Palo Alto, CA per 50 ml). Each extract was mixed with three volumes of ice-cold absolute ethanol, maintained at −20°C for 20 h and precipitated macromolecules were collected by centrifugation at 16,000*g* at 4°C for 20 min, washed with ice-cold absolute ethanol and air-dried prior to storage at −20°C until analysis. This protocol was applied to cartilage from three grade 0 patients (28 y, 47 y, and 64 y) and the same grade 3/4 pool used for CsCl gradient analysis (above).

ALKALI TREATMENT OF EXTRACTS

Portions of Extract 3 (see above) from the grade 3/4 pool, with or without prior Chase ABC digestion, were dried, dissolved in 10 μl of 0.2 M NaOH and maintained at room temperature for 2 h. Samples were neutralized by addition of 1 μl of 2 M HCl, then mixed with 15 μl of 1.6-fold concentrated gel-loading buffer[22] containing DTT (dithiothreitol) for Western blot analysis.

SYNOVIAL FLUID FRACTIONATION

Synovial fluid was obtained from 5 patients (52–75 y who were undergoing an IRB-approved protocol at Henry Ford Hospital) with knee effusions associated with degenerative arthritis having clinically required knee aspiration with or without injection. Patients with evidence of crystal disease or inflammatory arthritis such as gout were eliminated, as were patients who had received any joint injection within the preceding 3 months. Samples were pooled (~100 ml total) and adjusted to 4 M guanidine·HCl (with two tablets of Complete Mini©, Roche Palo Alto, CA) and a starting density of 1.5 g/ml with CsCl prior to ultracentrifugation. Fractions D1 (about 1.65 g/ml) through D6 (about 1.40 g/ml) were prepared, exhaustively dialyzed against water and dried under vacuum.

WESTERN BLOT ANALYSIS OF CARTILAGE EXTRACTS AND GRADIENT FRACTIONS

Portions of desalted and dried tissue extracts and CsCl gradient fractions were treated with glycosidases prior to gel electrophoresis as follows. For Chondroitinase ABC (Chase) digestion, samples were dissolved in 50 mM Na-acetate/50 mM Tris–HCl/5 mM EDTA/0.1 mM AEBSF, pH 7.6, and digested with proteinase-free Chase from *Proteus vulgaris* (Seikagaku, Inc.) (25 mU per 100 μg S-GAG (sulfated glycosaminoglycans)) at 37°C for 2 h. For hyaluronidase digestion, samples were resuspended in 100 mM ammonium acetate/5 mM EDTA/0.1 mM AEBS F ((2-aminoethyl)-benzenesulfonyl fluoride), pH 6.0 and digested at 60°C for 3 h with 0.5 TRU (turbidity reducing units) hyaluronidase (Hyase) from *Streptomyces hyalurolyticus* (Seikagaku, Inc.) per 100 μg S-GAG. Untreated samples were dissolved in the same buffers and incubated at the same temperature without deglycosidases. After incubation, samples were dried and dissolved in gel-loading buffer containing DTT before separation on SDS-PAGEs and Western blot analysis as described[22].

IMMUNOHISTOCHEMISTRY OF HUMAN CARTILAGES

Femoral condyle or tibial plateau cartilages were removed from the bone with a scalpel and rinsed in ice-cold PBS[25]. For one donor (45 y male with no history of joint disease, scored as grade 0[24]) cartilage was taken from one location (Site 1, medial notch, non-weight-bearing and showing smooth surface). For a second donor (63 y male with no history of joint disease, scored as grade 1) cartilage was removed from four sites on the femoral surface (Site 1, medial notch, non-weight-bearing and showing minor surface roughening; Site 2, center of patellar groove, showing advanced surface fibrillation; Site 3, lateral patellar groove with smooth surface; Site 4, lateral femoral condyle, weight bearing with smooth surface). For a third donor (72 y male undergoing total knee arthroplasty and scored as grade 4) tissue was taken from a central region of severely eroded medial tibial plateau cartilage as described in Ref. 22. Full depth, 5-mm diameter plugs were prepared, placed into 10% (v/v) neutral-buffered formalin for 48 h, followed by decalcification in 5% (w/v) EDTA in PBS for 14 days, before processing, paraffin embedding and sectioning as described. Immunohistochemistry was performed after deparaffinization as described[26]. Pretreatment of sections with Chase ABC or Streptomyces Hyase did not alter the intensity or distribution of immunoreactivity for any of the antibodies used. On the other hand, mild proteinase K digestion, commonly used for antigen retrieval[26], completely abolished immunoreactivity for all antibodies used here.