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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

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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

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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.

Table I
Primer sequences, annealing temperatures and amplicon sizes of cDNAs used to assess expression of Coll II, TSG-6, bikunin, ITIHCs-1, -2, and -3, and EF1α in human articular cartilage

Target	Primer sequence	Annealing temperature (°C)	Amplicon size
Coll II	S TGGGGCCTTGTTCACCTTTGA A CCGAGGCAACGATGGTCAGC	62	380
TSG-6	S ACCACAGAGAAGCACGGTCT A CAACTCTGCCCTTAGCCATC	62	164
Bikunin	S TACATCCATGGCCTGTGAGA A TTTATTTGGACCCAGGTTGC	60	395
ITIHC-1	S GACCCCAACACAGGCTTCTC A TTCCACACTCGGTGCAAAAC	62	304
ITIHC-2	S CGGCTCAAGTCACGAATCAG A TTGTAATCCCCGTCAATCAA	60	408
ITIHC-3	S CAGTGCTGCGCCTTATTCAG A ATGTGGCATCTGGCTTTGTG	62	485
EF1α	S AAAATGACCCACCAATGGAA A GCAGCATCACCAGACTTCAA	62	210

ARTICULAR CARTILAGE COLLECTION FOR PCR ASSAYS

Samples were collected from total knee arthroplasty tissues, in accordance with an IRB approval and categorized as "intact" or "fibrillated", based on the gross surface appearance. Cartilage was finely sliced, rinsed with PBS and pulverized under liquid nitrogen. RNA was extracted using a previously described protocol[33]. Briefly, 1–2 g of pulverized cartilage was homogenized in guanidinium isothiocyanate buffer, extracted with acidic phenol:chloroform, and precipitated with sodium acetate and isopropanol. The precipitate was processed through an "RNA clean-up" protocol (Invitrogen) with on-column DNase treatment. RNA was eluted with DEPC (diethylpyrocarbonate) -treated water and 0.5 μg of RNA from each sample was electrophoresed through formaldehyde agarose gels and stained with ethidium bromide to verify ribosomal RNA integrity. One microgram of RNA was reverse-transcribed using oligo T primers (Superscript II first strand synthesis kit, Invitrogen) and 1 μl of the product used for PCR. Primers were designed using Primer3 version 0.4.0 software[34]. Primer sequences, annealing temperatures and amplicon sizes for each reaction are provided in Table I. All reactions involved an initial 3-min 95°C denaturation step, 15-s annealing times and 20-s extension times, and were continued for 30–45 cycles. Collagen type II (Coll II) was assayed as a chondrocyte-specific marker, while elongation factor 1 alpha (EF1α) was used to demonstrate approximately equal mRNA quantities. RNAs from human cell lines were used to optimize PCR conditions and as positive controls for the reactions (Fig. 12). Reactions lacking RT template were used as negative controls. The PCR amplicons were cloned and sequenced to verify cDNA identities. Aliquots (20 μl) of each PCR reaction were electrophoresed in 2.0% agarose gels, stained with ethidium bromide and photographed using a Kodak EDAS 290 gel-imaging system.

Results

CHARACTERIZATION OF BIKUNIN, HC1 AND HC2 IN CARTILAGE EXTRACTS

Cartilage pools from normal donors (grades 0–1) and OA patients (grades 3–4) were extracted with 4 M guanidine·HCl and fractionated by CsCl gradient centrifugation. Fractions D1 through D6 from each group were analyzed by Western blotting with antibodies for bikunin (Fig. 1), HC1 (Fig. 2), HC2 (Fig. 3); data for untreated samples

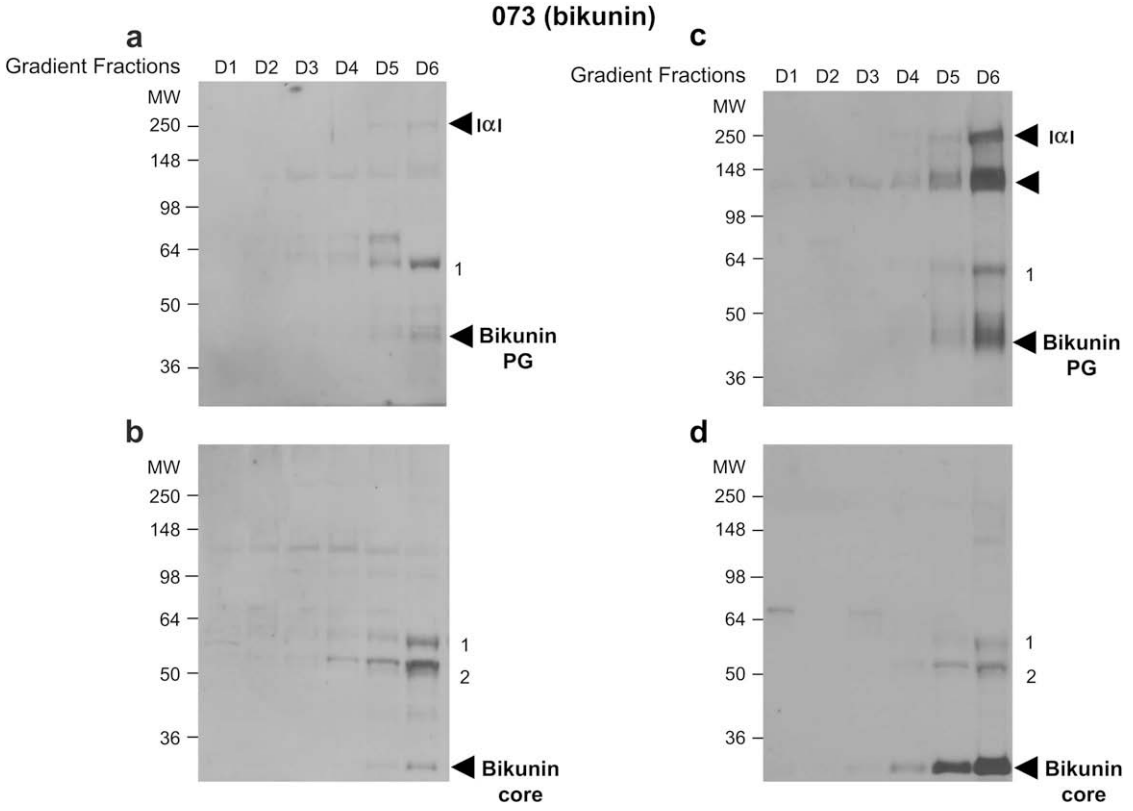

Fig. 1. Western blot analyses of bikunin in CsCl gradient fractions prepared from guanidine·HCl extracts of normal (grades 0–1) and OA (grades 3–4) human cartilage. Fractions from normal cartilages (left) and OA cartilages (right) were analyzed for bikunin with antibody 073, without (panels a and c) and with (panels b and d) Chase ABC digestion. The portions loaded corresponded to about 2 mg wet wt. of cartilage for both normal and OA samples.

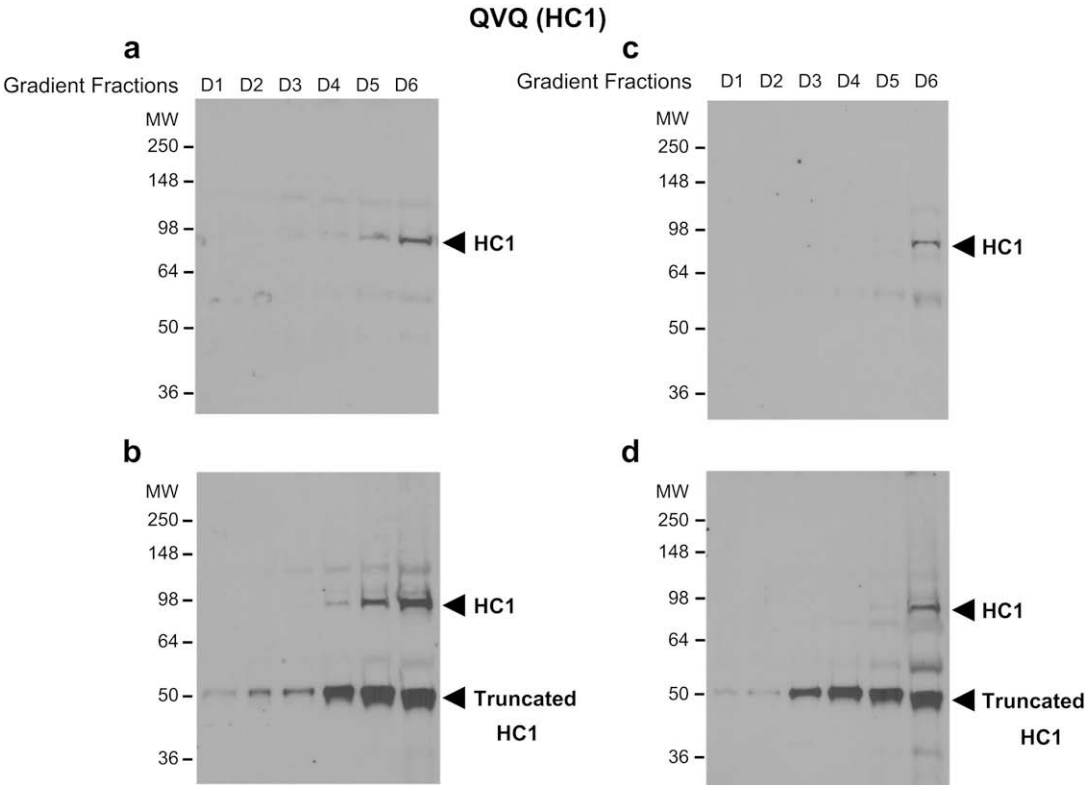

Fig. 2. Western blot analyses of HC1 in CsCl gradient fractions prepared from guanidine·HCl extracts of normal (grades 0–1) and OA (grades 3–4) human cartilage. Fractions from normal cartilages (left) and OA cartilages (right) were analyzed for HC1 with antibody QVQ, without (panels a and c) and with (panels b and d) Chase ABC digestion. The portions loaded corresponded to about 2 mg wet wt. of cartilage for both normal and OA samples.

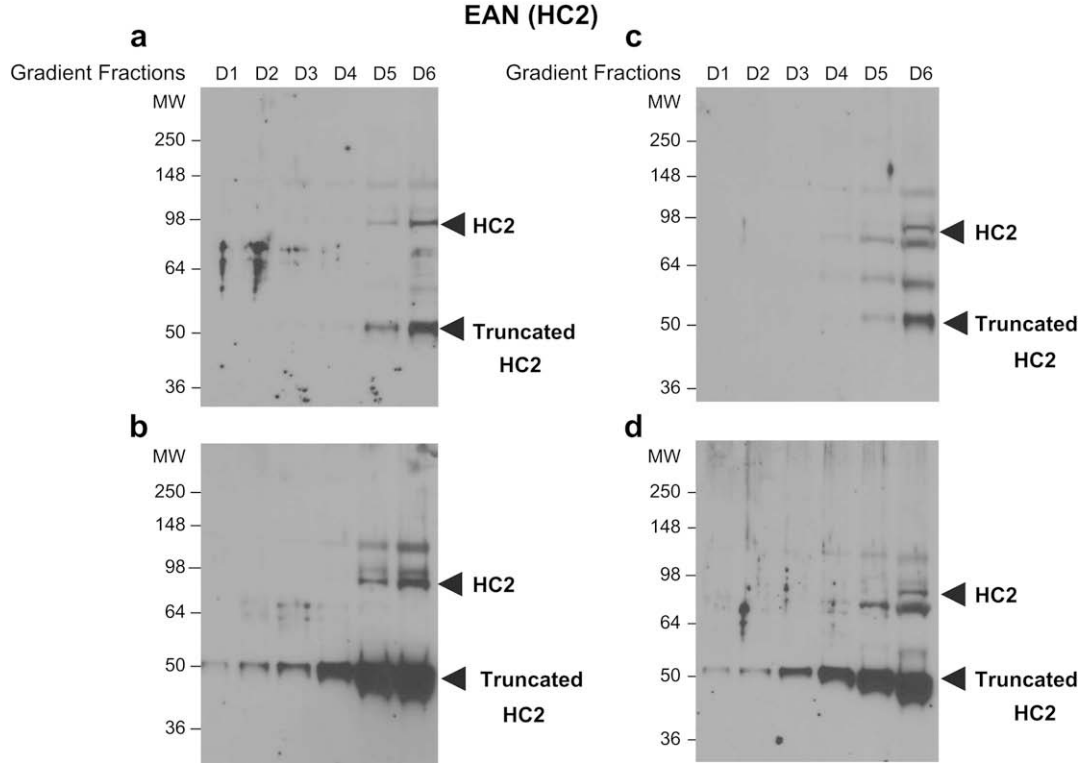

Fig. 3. Western blot analyses of HC2 in CsCl gradient fractions prepared from guanidine·HCl extracts of normal (grades 0–1) and OA (grades 3–4) human cartilage. Fractions from normal cartilages (left) and OA cartilages (right) were analyzed for HC2 with antibody EAN, without (panels a and c) and with (panels b and d) Chase ABC digestion. The portions loaded corresponded to about 2 mg wet wt. of cartilage for both normal and OA samples.

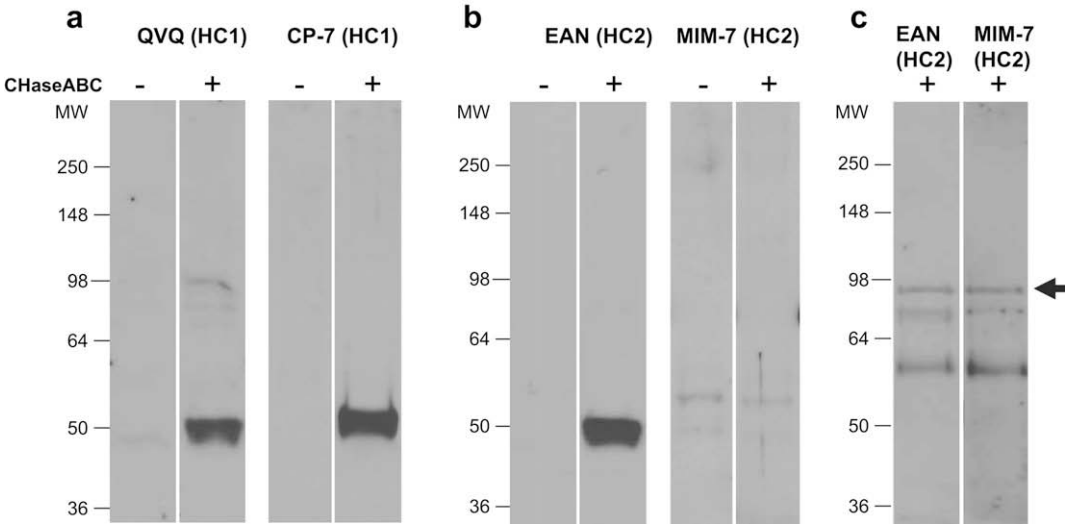

Fig. 4. Western blot analyses of HC1 and HC2 with multiple antibodies. Portions of guanidine·HCl extracts (sequential Extract 3) of OA cartilage were analyzed for HC1 with antibodies QVQ and CP7 (panel a), and for HC2 with antibodies EAN and MIM-7 (panel b), both without (−) and with (+) Chase ABC digestion. Portions of the CsCl fraction D4 of OA synovial fluids were analyzed with EAN and MIM-7 for HC2 with (+) Chase ABC digestion. The portions loaded corresponded to about 2.5 mg wet wt. of cartilage or 8.5 μl synovial fluid.

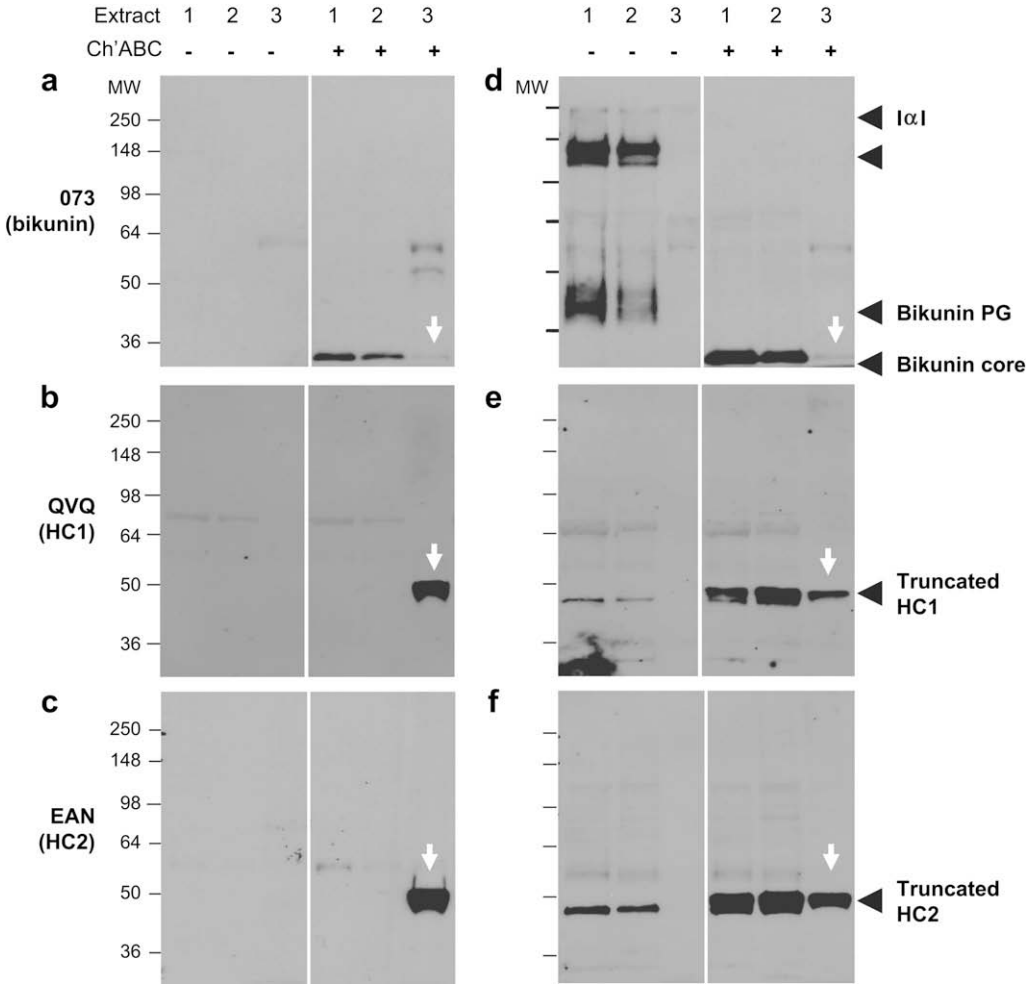

Fig. 5. Western blot analyses of associative and dissociative extracts from normal and OA cartilages. Portions of sequential Extracts 1−3 (see Methods for details) from normal (panels a−c) and OA (panels d−f) cartilage were analyzed for bikunin with antibody 073 (panels a and d), HC1 with antibody QVQ (panels b and e) and HC2 with antibody EAN (panels c and f), without (−) and with (+) Chase ABC digestion. The portions loaded corresponded to about 5 mg wet wt. of cartilage for both normal and OA samples.

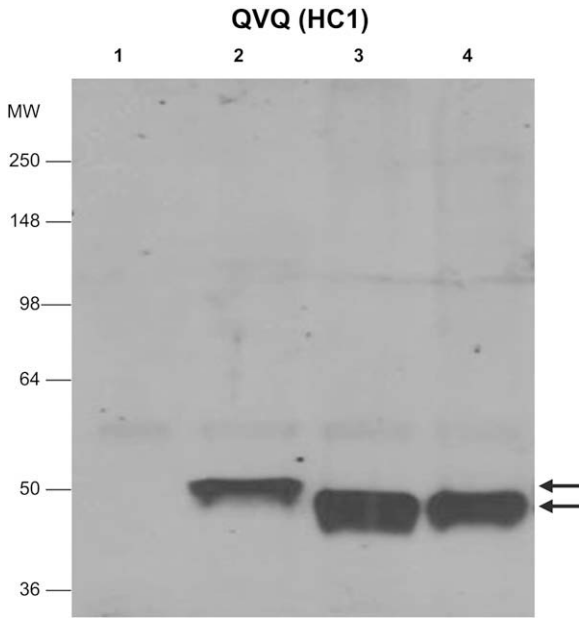

Fig. 6. Release of HC1 by treatment of extracts of normal cartilage with Chase ABC or alkali. Portions of guanidine·HCl extracts (sequential Extract 3) of normal cartilage were analyzed for HC1 (with antibody QVQ) when untreated (lane 1), digested with Chase ABC (lane 2), alkali treated (lane 3) or after both Chase ABC and alkali treatment (lane 4). The portions loaded corresponded to about 2.5 mg wet wt. of cartilage.

(panels a and c) and samples digested with Chase ABC (b and d) are shown in each case. The 073 antibody revealed that extracts of normal cartilage contained trace amounts of bikunin·CS (~45 kDa) in the D5 and D6 fractions [Fig. 1(a)], and this species was converted to the corresponding ~30-kDa core protein after Chase treatment [Fig. 1(b)]. In addition, both low buoyant density fractions contained an immunoreactive species migrating with a molecular weight of ~250 kDa, and its removal after Chase treatment is in keeping with it representing intact IαI[12,27]. In extracts from OA cartilage both the bikunin·CS and the IαI were significantly more abundant in fractions D5 and D6 compared to normal cartilage [Fig. 1(c)] with an additional chondroitinase-sensitive species migrating with a molecular weight of 130 kDa [Fig. 1(c, d)]. The latter has a molecular weight similar to pre-alpha-inhibitor (PαI: bikunin·HC3), however, Western blot analysis of these samples with HC3-specific antibodies from two different sources showed no immunoreactivity (data not shown). The bikunin-positive species migrating at ~60 kDa [Fig. 1(a–d), band 1, Chase ABC-insensitive] and ~55 kDa [Fig. 1(b, d); band 2, Chase ABC-generated], were not characterized but were also detected using the CP6 antiserum, raised against the N-terminus of bikunin (data not shown).

With respect to HC1 and HC2, use of antibodies QVQ and EAN, respectively, showed that both normal and OA cartilage extracts contained a small amount of unbound, full-length HCs (~90 kDa)[12,13,28], in the D5 and D6 fractions [Figs. 2(a, c) and 3(a, c)]. Digestion of both normal and OA gradient fractions with Chase ABC-generated abundant HC1 and HC2 species, which migrated with unexpectedly low molecular weights of about 50 kDa [Fig. 2(b, d), truncated HC1; Fig. 3(b, d), truncated HC2]. Such short HC2 is also detectable prior to Chase ABC treatment. The proportions of full-length and truncated forms of HC1 and HC2 appeared to be similar for the two HCs (perhaps 1:20) with little difference in protein levels between normal and OA samples. However, the short forms of HC1 and HC2 were the dominant species attached to GAG (glycosaminoglycan), and thus released by Chase ABC. The identities of the minor HC1-positive species at about 55 kDa [Fig. 2(d)] and the HC2-positive species at about 130 kDa [Fig. 3(b)], 55 kDa and 75 kDa [Fig. 3(d)] are unknown. Notably, both the 55 kDa and 75 kDa HC2-positive species were also recognized by the MIM-7 antiserum, raised against the C-terminus of HC2 (data not shown).

ANALYSIS OF TRUNCATED FORMS OF HC1 AND HC2 IN CARTILAGE EXTRACTS

To further define the composition of the truncated HCs in OA cartilage extracts, samples were analyzed by Western blotting with two HC1-specific antibodies (QVQ and CP7) and three HC2-specific antibodies (EAN, CP10 and MIM-7) (see Methods and Supplementary Fig. 1, for details). We found that truncated HC1 reacted with both QVQ and the N-terminus-specific CP7 [Fig. 4(a)]. However, the truncated HC2 reacted with anti-EAN but not with the C-terminus-specific MIM-7 [Fig. 4(b)] or the N-terminus-specific CP10 (data not shown). This suggests that truncated HC2 lacks the C-terminal region and the N-terminus, whereas truncated HC1 lacks only the C-terminal region. This interpretation for HC2 is supported by the fact that both EAN and MIM-7 did detect the 90 kDa form of HC2 in Chase ABC-digested portions of CsCl fractionated OA synovial fluids [Fig. 4(c), arrow]. With respect to the 50 kDa forms, it is significant that antisera to the N-terminus of HC1 (CP7) and HC2 (CP10) also detect 50-kDa species in preparations of IαI purified from human serum, whereas MIM-7 does not (see Supplementary Fig. 1). The 50 kDa species in serum and cartilage may not be identical, particularly since only the cartilage species are CS-bound and the CP10 antibody detects HC2 in serum but not in cartilage extracts. CP10 did, however, react with a 60 kDa form of HC2 in CsCl gradient fractions of OA cartilage and synovial fluids (data not shown) and also cell cluster-associated HC2 on immunohistochemistry (Fig. 10).

RECOVERY OF BIKUNIN AND HCs IN EXTRACTS OF NORMAL AND OA CARTILAGES

To explore the extracellular associations of bikunin, HC1, HC2 in normal and OA cartilages, we applied a three-step sequential extraction protocol (see Methods for details) followed by Western blot analysis without and with Chase ABC digestion (Fig. 5). The data demonstrated that IαI, the putative PαI (or bikunin with a single HC) (at 130 kDa), and the bikunin·CS (at 45 kDa) are essentially present only in OA tissue [Fig. 5(d)]. In addition the analyses showed that these species are quantitatively extracted by isotonic salt (Extract 1) or isotonic salt plus detergent (Extract 2) and did not require 4 M guanidine·HCl (Extract 3) for solubilization. The molecular basis for the doublet of bikunin-positive material at 120–140 kDa in unfractionated Extracts 1 and 2 from OA cartilage [Fig. 5(d)] is unknown. However, on long exposure of the anti-HC1 (QVQ) and anti-HC2 (EAN) blots of such extracts, it was observed that the HC antibodies reacted only with the faster migrating band. Since there was no evidence for unprocessed, full-length HCs in these samples, this may represent bikunin·CS substituted with truncated HCs. This also suggests that the more abundant, slower band represents PαI [bikunin·HC3,

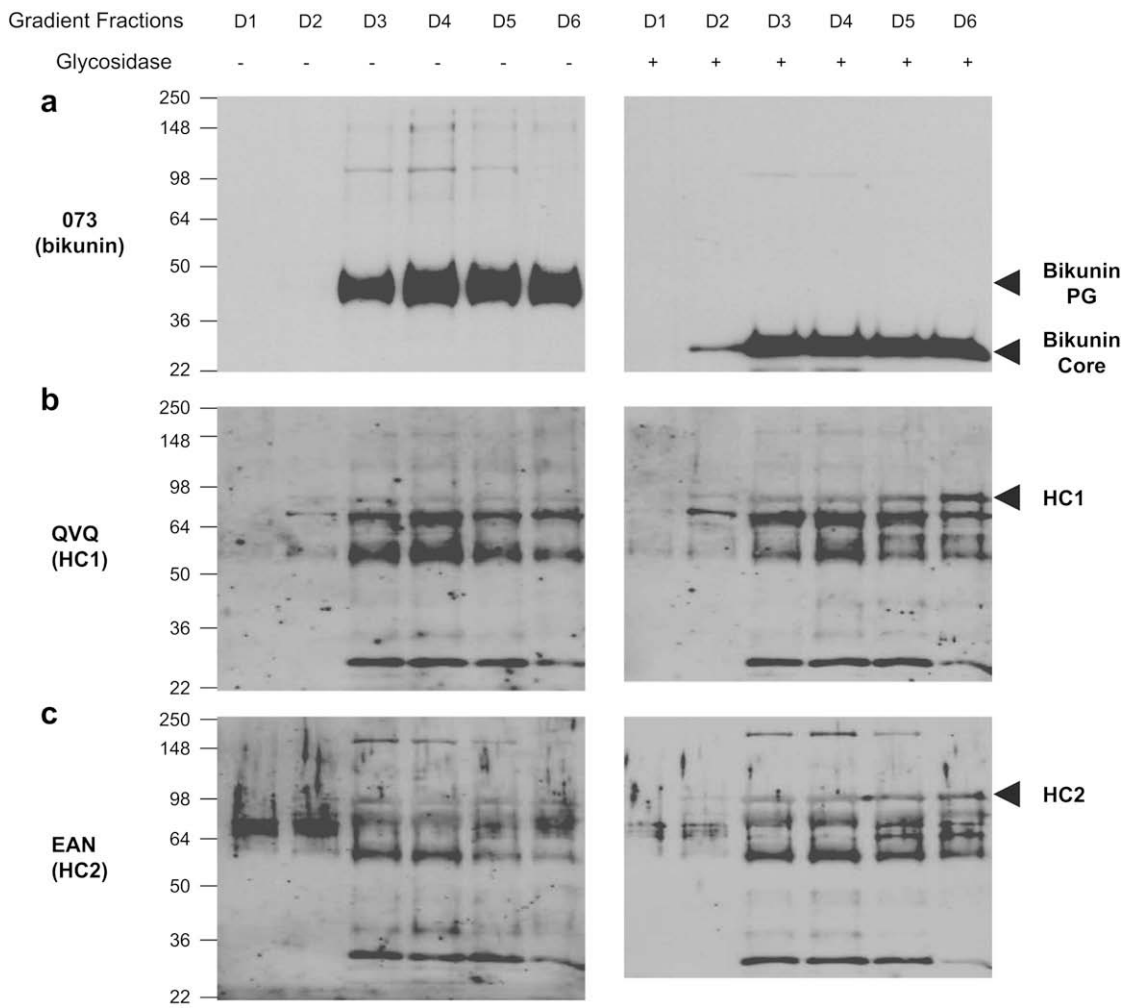

Fig. 7. Western blot analyses of CsCl gradient fractions generated from osteoarthritic synovial fluid pool. Fractions were analyzed for bikunin (panel a), HC1 (panel b) and HC2 (panel c) both without (−) and with (+) Chase ABC digestion (panel a) or without (−) and with (+) Streptomyces Hyase digestion (panels b and c). The portions loaded corresponded to about 2.5 μl of synovial fluid for bikunin and about 8.5 μl of synovial fluid for HC1 and HC2. The doublet at 64 kDa and 68 kDa (most evident on the HC2 blots) appears to be non-specific, since we have detected it on blots with many antibodies unrelated to this study.

see Fig. 1(c) and text]. The finding that the 250 kDa IαI was barely detectable in these unfractionated extracts of OA cartilage (but readily detected in the D6 of CsCl gradient fractions, [Fig. 1(c)]) may be due to an association of IαI with other components which may have prevented it from entering the gel or resulted in low immunoreactivity due to masking on the membrane.

TRUNCATED HCs ARE PRIMARILY LINKED TO A CSPG IN CARTILAGE EXTRACTS

The covalent linkage of truncated HCs to HA or CS *via* ester bonds (Figs. 2–5) in both normal and OA cartilages, was further supported by the alkali sensitivity of the HC1 complex in Extract 3 material (Fig. 6). The 50 kDa species was absent from untreated extracts (lane 1), but was generated by Chase ABC digestion (lane 2), alkali treatment (lane 3) or by a combination of both treatments (lane 4). Digestion of such complexes from normal and OA samples with *Streptomyces* Hyase (under conditions which quantitatively degraded uncomplexed HA or HA in A1-aggrecan-aggregates), did not, however, generate detectable "free" HCs (data not shown) suggesting that HC·HA complexes are not abundant in cartilage. In addition, when samples were treated with keratanase II or endo-β-galactosidase (or both enzymes), there was no marked change in HC2 or bikunin migration properties (data not shown). The data illustrated in Fig. 6 also indicate that the apparent doublet corresponding to truncated HCs [see also Fig. 5(e, f)] may be explained by variable O-linked glycosylation since Chase ABC digestion alone generated a slower migrating band than that produced by treatment with alkali.

The HCs on circulating IαI are attached to bikunin·CS by ester linkages[11]. However, analysis of the distribution of HCs and bikunin in cartilage extracts suggested that a significant proportion of HCs, particularly in normal cartilages, is present on a CSPG other than bikunin. This was readily seen on analysis of CsCl gradient fractions of normal cartilage where fractions containing abundant HC1 and HC2 [Figs. 2(b) and 3(b), fractions D4 and D3] were apparently devoid of bikunin [see Fig. 1(b)], a conclusion which was also supported by extended exposure of the Western for bikunin. Analysis of the D3 fraction from OA cartilage [Figs. 1(d), 2(d) and 3(d)] was also consistent with this

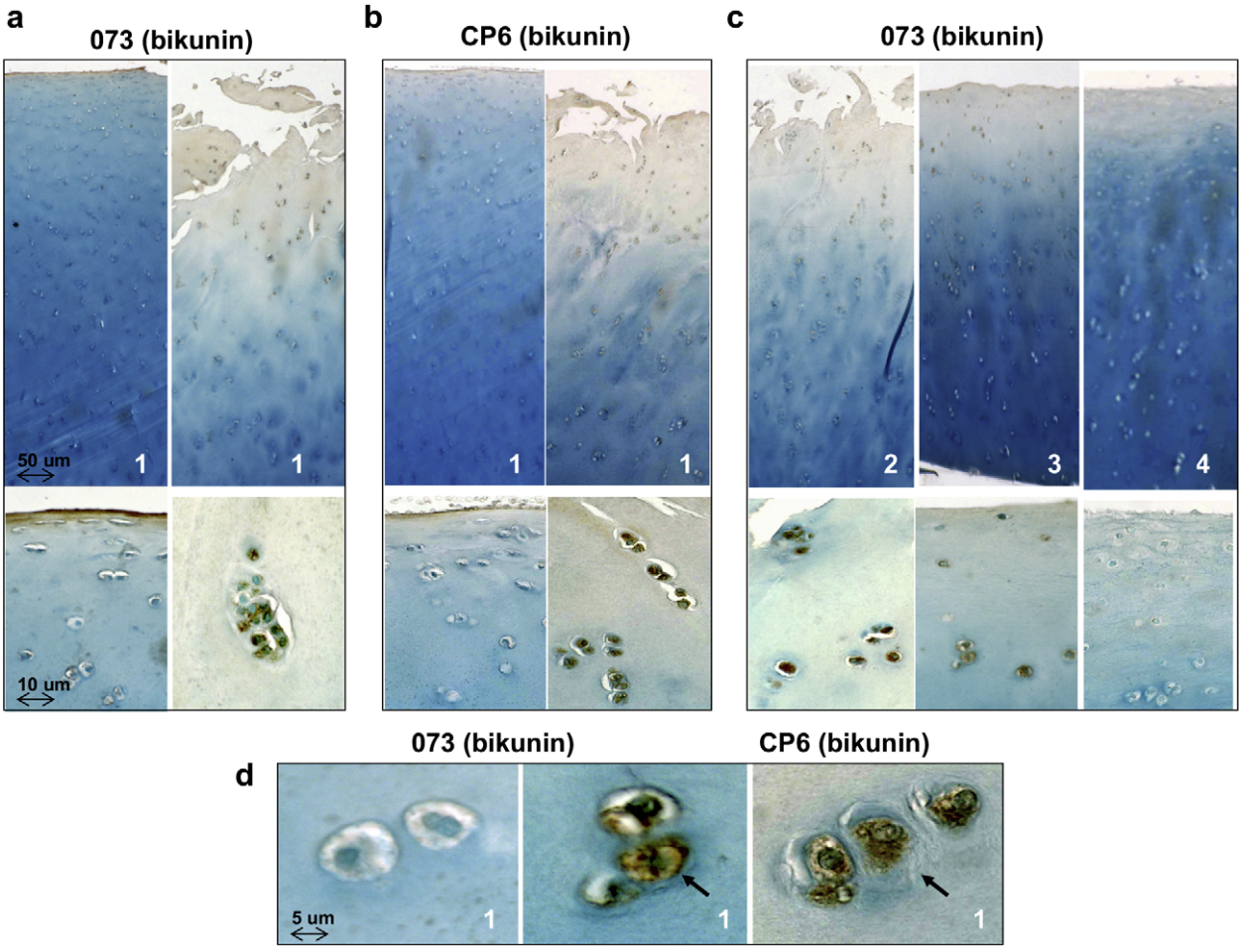

Fig. 8. Immunohistochemical localization of bikunin in femoral condylar cartilages. Cartilage from the medial notch of a normal 45 y male and four separate sites on the femoral surface of a 63 y male with no history of joint disease (see Methods for details of sites and gross appearance of cartilage surfaces) were processed for immunohistochemical staining for bikunin. Images were taken at 4× and 20× magnification (upper and lower images in panels a–c, respectively) or 40× (panel d). Panel a (left), Site 1 from 45 y male and panel a (right), Site 1 from 63 y male both stained with 073. Panel b (left), Site 1 from 45 y male and panel b (right), Site 1 from 63 y male stained with CP6. Panel c, Sites 2–4 were stained with 073. Panel d (left), cell-associated staining in Site 1 from 45 y male. Panel d (middle and right), cell-associated staining in Site 1 from 63 y male. Antibodies were 073 (left and middle) and CP6 (right).

interpretation. Most strikingly, Chase ABC-digested Extract 3 from both normal and OA cartilages (Fig. 5, see white arrows) contained abundant HC1 and HC2, but essentially undetectable bikunin, even after extended film exposure.

ANALYSIS OF OSTEOARTHRITIC SYNOVIAL FLUIDS

To determine whether the bikunin and HC complexes identified in OA cartilage were deposited after diffusion from the synovial fluid, we examined fluid from outpatients undergoing clinically required joint drainage. Pooled fluids were subjected to dissociative CsCl gradient centrifugation (starting density 1.5 g/ml) and fractions D1 through D6 were digested with either Chase ABC or *Streptomyces* Hyase, followed by Western analysis (Fig. 7). The D3 to D6 fractions contained abundant bikunin·CS at 45 kDa [Fig. 7(a), left-hand panel], which was converted to 30 kDa bikunin core protein [Fig. 7(a), right-hand panel] following Chase ABC digestion. There was, however, no evidence for IαI complexes [see Fig. 1(c), for example] in the OA synovial fluid pool, suggesting that the abundant bikunin·CS [Fig. 7(a)] represents a by-product generated by TSG-6-mediated transfer of HCs from serum- or joint tissue-derived IαI onto HA in the joint space of these individuals. Consistent with this was the finding that full-length HC1 and HC2 [90 kDa, Fig. 7(b, c), right-hand panels] were released after *Streptomyces* Hyase digestion of the D5 and D6 fractions. Interestingly, the truncated HCs were not detectable in these fractions either before or after Hyase treatment. In addition, no truncated HCs, and only trace amounts of the 90 kDa HCs, were generated by Chase ABC digestion (data not shown). The identity of the other major species at 25–30 kDa, 55 kDa and 75 kDa detected by the HC1- and HC2-specific antibodies [Fig. 7(b, c)] is unknown, however, the 55 kDa and 75 kDa species also appear to be present in extracts of OA cartilages (see Figs. 2 and 3) and they might represent HC degradation products.

IMMUNOHISTOCHEMICAL LOCALIZATION OF BIKUNIN, HC1 AND HC2 IN NORMAL AND OA CARTILAGES

As the biochemical data described above were obtained from cartilage pools (five normal donors and seven OA

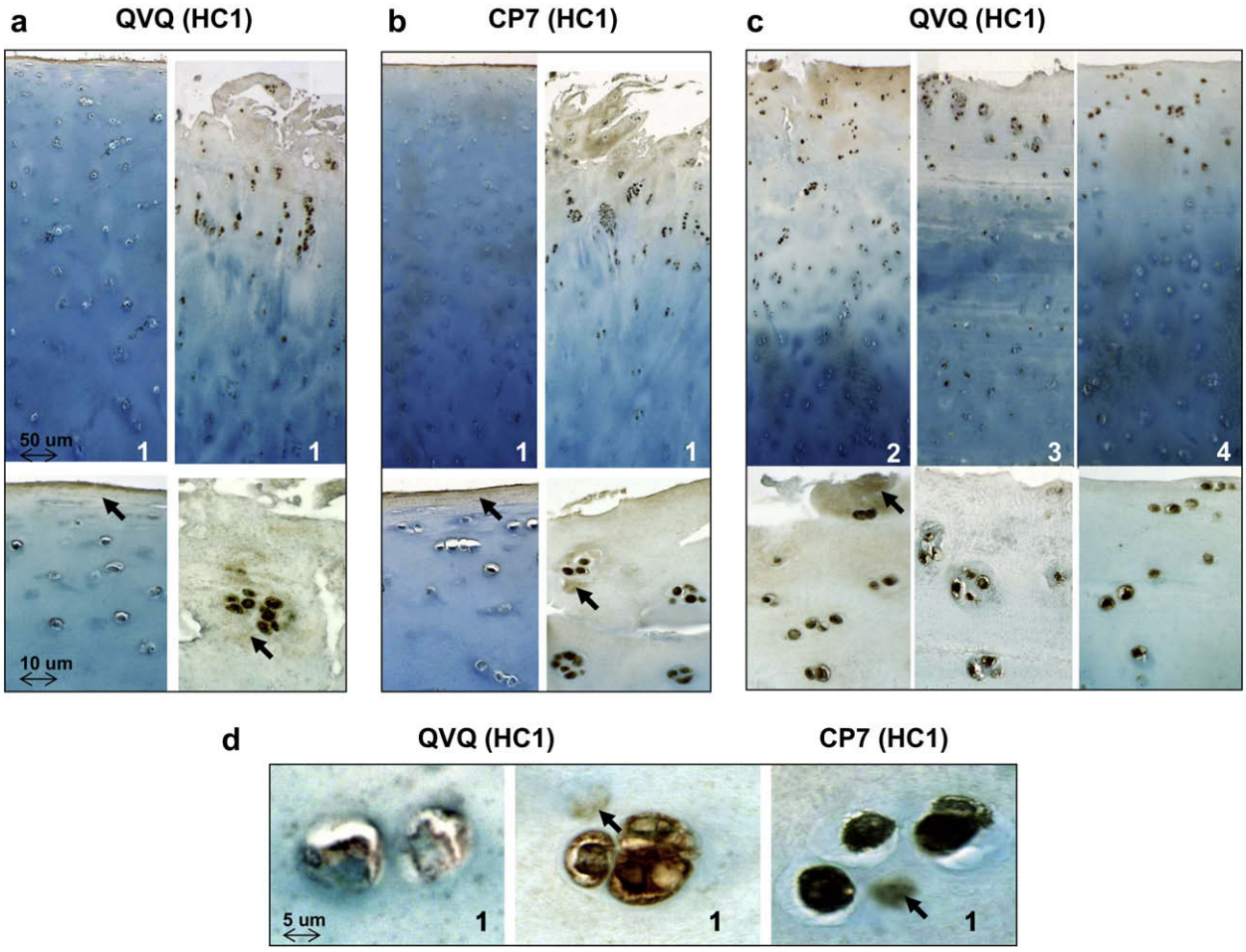

Fig. 9. Immunohistochemical localization of HC1 in femoral condylar cartilages. Cartilage from the medial notch of a normal 45 y male and four separate sites on the femoral surface of a 63 y male with no history of joint disease (see Methods for details of sites and gross appearance of cartilage surfaces) were processed for immunohistochemical staining for HC1. Images were taken at 4× and 20× magnification (upper and lower images in panels a–c, respectively) or 40× (panel d). Panel a (left), Site 1 from 45 y male and panel a (right), Site 1 from 63 y male stained with QVQ. Panel b (left), Site 1 from 45 y male and panel b (right), Site 1 from 63 y male stained with CP7. Panel c, Sites 2–4 stained with QVQ. Panel d (left), cell-associated staining in Site 1 from 45 y male. Panel d (middle and right), cell-associated staining in Site 1 from 63 y male. Antibodies were QVQ (left and middle) and CP7 (right).

patients) it provides no information on the distribution of bikunin and HCs in cartilages of different histopathological appearance; in addition the extraction data do not indicate whether the resident chondrocytes might be the biosynthetic source of bikunin and HCs. Therefore, immunohistochemical staining for bikunin (Fig. 8), HC1 (Fig. 9) and HC2 (Fig. 10) was done on sections of different histopathological appearance from the normal 45 y male donor (Site 1), the normal 63-y-old male donor (Sites 1–4) and the 72 y male donor undergoing joint replacement for OA (Fig. 11).

For bikunin, a comparison of the location-matched Site 1 samples [Fig. 8(a)] showed that the section with a smooth surface (left) showed no specific staining in either cells or matrix, whereas the section with a fibrillated surface (right) showed intense staining of cell groups in the fibrillated regions. Furthermore, specificity of the immunostaining for bikunin with 073 was verified by use of a second antibody CP6 [Fig. 8(b)]. The presence of cell-associated bikunin near fibrillated surfaces was confirmed with Site 2 sections [Fig. 8(c)]. Tissue from Sites 3 and 4, [Fig. 8(c)] showed that in some regions with a macroscopically smooth surface, isolated cells or groups of cells in the superficial regions stained weakly positive for bikunin. High-magnification images of staining in cell groups from the two Site 1 samples showed that for both antibodies the cell-associated and intracellular staining [Fig. 8(d), arrows] was most noticeable around cell groups that also showed intense methyl-green (anionic) staining in the cell-associated matrix. This suggests that increased labeling for bikunin is associated with increased *de novo* PG/GAG production in damaged regions of cartilage.

For HC1, individual cells in the superficial region of all sections with a smooth surface were stained at a low or medium intensity [Fig. 9(a, b), left-hand panels and Fig. 9(c), right-hand panel] but there was no detectable staining in the transitional or radial zones of the same tissue section (see Ref. 29 for definition of cartilage zones). On the other hand, there were marked increases in cell-associated staining in the superficial region of all sites with a fibrillated surface, whether the fibrillation was slight or deep [Fig. 9(a, b), right-hand panels and Fig. 9(c), two left-hand panels]. Specificity of staining for HC1 with QVQ was also confirmed

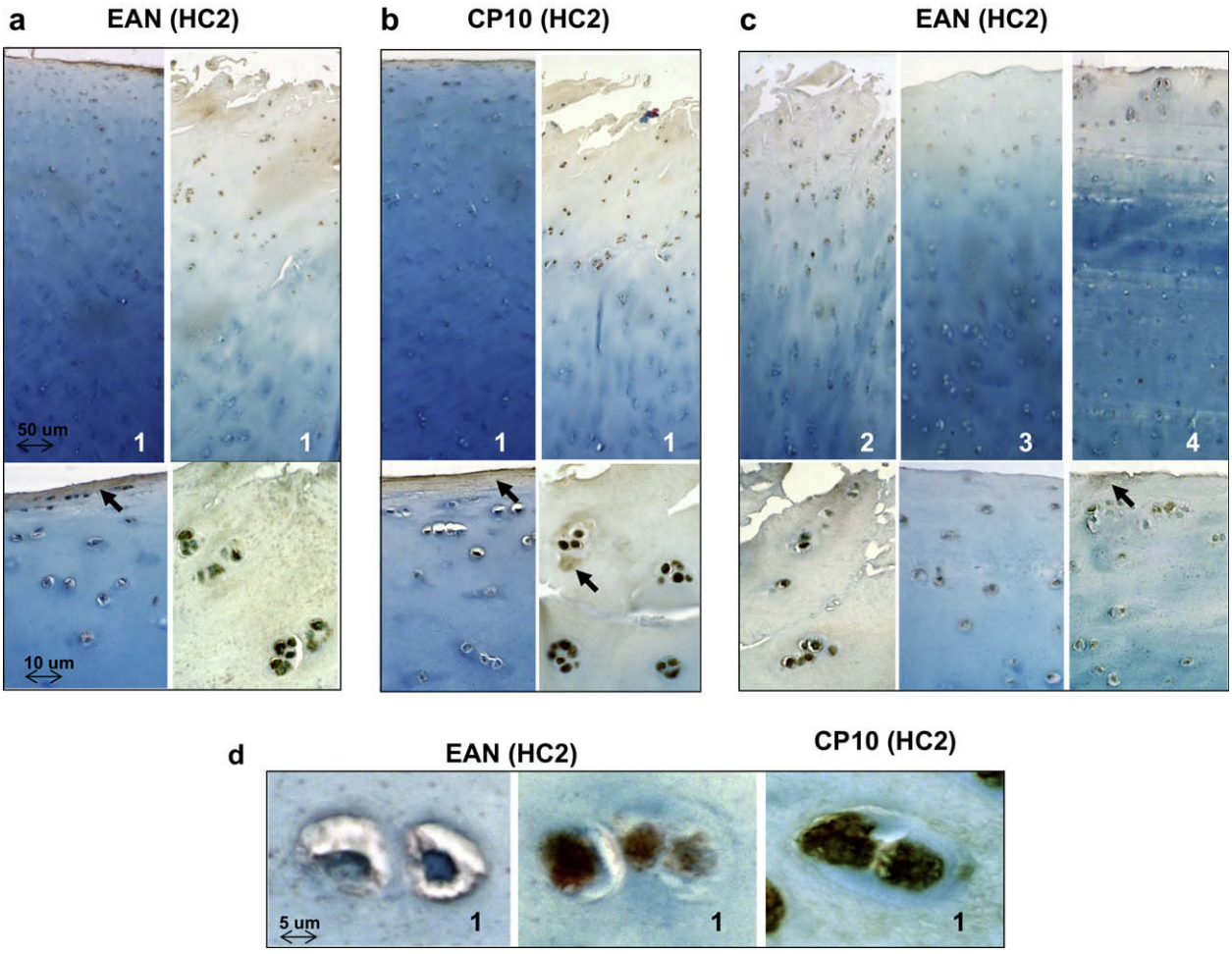

Fig. 10. Immunohistochemical localization of HC2 in femoral condylar cartilages. Cartilage from the medial notch of a normal 45 y male and four separate sites on the femoral surface of a 63 y male with no history of joint disease (see Methods for details of sites and gross appearance of cartilage surfaces) were processed for immunohistochemical staining for HC2. Images were taken at 4× and 20× magnification (upper and lower images in panels a–c, respectively) or 40× (panel d). Panel a (left), Site 1 from 45 y male and panel a (right), Site 1 from 63 y male stained with EAN. Panel b (left), Site 1 from 45 y male and panel b (right), Site 1 from 63 y male stained with CP10. Panel c, Sites 2–4 stained with EAN. Panel d (left), cell-associated staining in Site 1 from 45 y male. Panel d (middle and right), cell-associated staining in Site 1 from 63 y male. Antibodies were EAN (left and middle) and CP10 (right).

by use of a second antibody, CP7 [Fig. 9(b)]. In addition to the cellular staining, some regions of the surface, the underlying matrix and the inter-territorial matrix around cell groups showed a diffuse positive staining pattern with both antibodies (see black arrows on panels a–c). High-magnification images [Fig. 9(d)] of staining in cell groups from the two Site 1 samples confirmed that staining with both antibodies appeared to be primarily intracellular with some reactivity at the margins of the cell-associated matrix (see arrows).

For HC2, individual cells in the superficial region of all sections with a smooth surface stained at low intensity [Fig. 10(a, b), left-hand panels and Fig. 10(c), right panels]. As for HC1, there was a marked increase in cell-associated staining in the superficial region of all sites with a fibrillated surface [Fig. 10(a–c)]. Also similar to HC1, there was no staining in the transitional or radial zones, but some matrix staining at the surface and around cell groups with both HC2 antibodies (see black arrows). Specificity of staining for HC2 with EAN was confirmed with a second antibody, CP10 [Fig. 9(b)]. High-magnification images of cells from the two Site 1 samples showed that HC2 staining with both antibodies appeared to be predominantly intracellular, even in cells that were undergoing mitosis [Fig. 10(d), right-hand panel]. In addition, the finding that the staining pattern for HC1 and HC2 was confined to the superficial zone of Site 3 (63 y male) was supported by Western analysis of extracts of the individual zones (data not shown).

For cartilages taken from the late-stage OA joints, two distinct staining patterns were observed (Fig. 11). Firstly, in regions with a deeply fissured cartilage surface, cell groups in the superficial zone, and adjacent to fissures, stained positive for HC1 and HC2, [Fig. 11(b, c), left-hand panels], but bikunin staining was virtually absent [Fig. 11(a), left-hand panel]. Secondly, other areas of cartilaginous tissues taken from the same tibial plateau, showed less apparent surface damage [Fig. 11(a–c), right-hand panels] and these had a thick superficial layer of cartilaginous matrix, in which all three components (bikunin, HC1 and HC2) were markedly stained, both throughout the matrix and associated with the cells (high-magnification images, panels a–c). It is possible that this intensely-stained

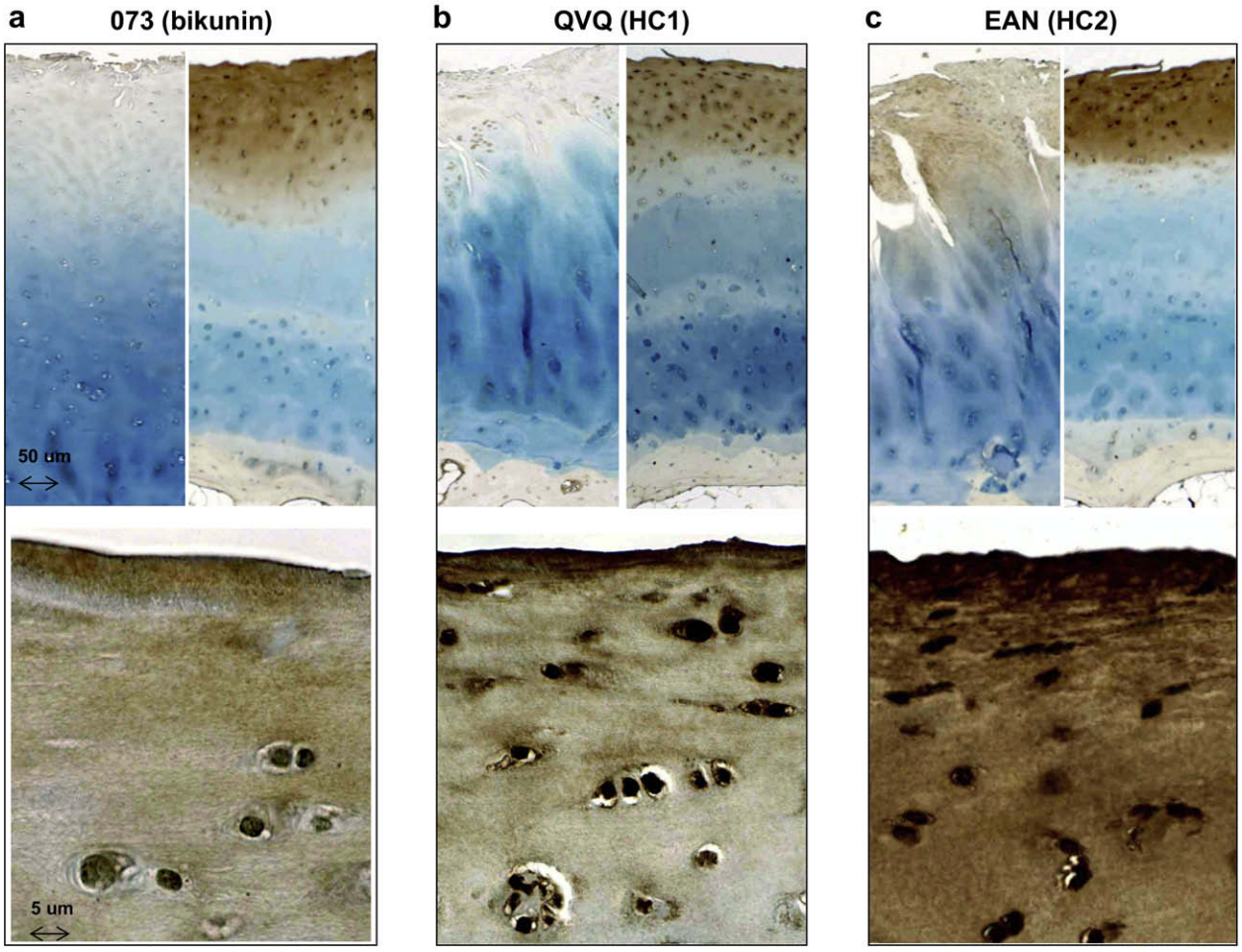

Fig. 11. Immunohistochemical localization of bikunin, HC1 and HC2 in late-stage osteoarthritic human tibial plateau cartilage. Full depth cartilage from the tibial plateau of a 72 y male undergoing joint replacement (see Methods for details) was processed for immunohistochemical staining for bikunin (antibody 073, panel a), HC1 (antibody QVQ, panel b) and HC2 (antibody EAN, panel c). Images were taken at 4× and 20× magnification (upper and lower images in panels a–c, respectively). Two markedly different tissue morphologies and staining patterns were observed. The left-hand panels of a–c show the appearance in areas with gross surface fibrillation. The right-hand panels of a–c and the high-magnification images below show the appearance in areas with an intact and apparently overgrown surface.

tissue represents a *de novo* cartilaginous overgrowth from a reparative attempt, and that it contains proteins originating from the resident tissue cells as well as some derived by diffusion from the synovial fluid. This form of intensely-stained matrix may explain our observations (Fig. 5), that a pool of associatively extractable bikunin–HC complexes are much more abundant in OA cartilage than normal cartilages.

ANALYSIS OF HUMAN CARTILAGES FOR mRNAs FOR BIKUNIN, HCs AND TSG-6

To determine whether human cartilages have the capacity to synthesize bikunin, HC1, HC2, HC3 and TSG-6 (the mediator for HC transfer to HA from CS), we did RT-PCR analysis for these transcripts in cartilage samples (from six separate patients) which on gross examination had an intact glossy surface or a fibrillated surface (Fig. 12). Collagen II transcripts were analyzed as a positive cartilage control and EF1α as an internal loading control. We found clear evidence for the presence of HC1, HC2, HC3 and TSG-6 transcripts with no consistent differences between the cartilage types. On the other hand we were unable to detect transcripts for bikunin.

Discussion

Our data suggest strongly that HC1 and HC2 are synthesized by human articular chondrocytes in a novel truncated form attached to a CSPG other than bikunin. On the other hand, the bikunin present in cartilage extracts (much more abundant in OA tissue and associated with cells near fibrillated surfaces) appears to be derived from the circulation by diffusion from the synovial fluid into the cartilage. Our Western analysis of HC1 and HC2 suggests that the truncated forms are present in similar abundance in normal and advanced OA tissues which appears to conflict with a recent proteomic analysis[30] reporting a massive increase in HC2 in OA tissues; this difference might, however, be explained by patient and donor tissue selection.

The presence of truncated HCs in human cartilage may be due to alternate mRNA splicing or a novel upstream truncation of the proteins during Golgi processing[18]. If, as seems likely, the addition of these 50 kDa HCs to CS occurs by addition to a C-terminal aspartic acid (as occurs with extended HCs[18]), then this would require that chondrocyte HCs be cleaved (by what is thought to be an autocatalytic event) at a consensus Asp-X-Asp-Pro site(s)[18] during

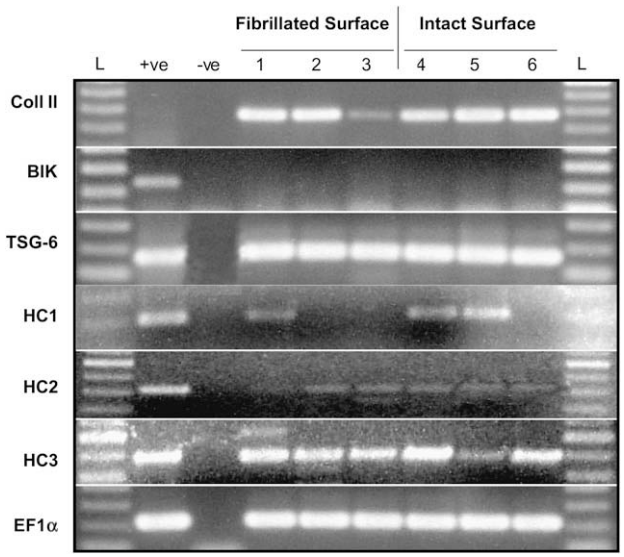

Fig. 12. RT-PCR amplification of Coll II, bikunin (BIK), TSG-6, ITIHCs-1 (HC1), -2 (HC2) and -3 (HC3), and EF1α mRNAs in human articular cartilage samples. Details of the PCR primers and reaction conditions are provided in Table I and Methods. Six separate patients were analyzed and results are shown for mRNA isolated from cartilages with a fibrillated surface (lanes 1–3) or an intact surface (lanes 4–6).

biosynthesis. Such sites might be at $DGD_{405}P_{406}$ in HC1 (the full-length product is cleaved at $DTD_{672}P_{673}$) and at $DGD_{423}P_{424}$ in HC2 (the full-length product is cleaved at $END_{702}P_{703}$). In this regard, structural studies of IαI purified from human serum[31] have shown that they contain quite large amounts of 50 kDa HC1 and HC2, with similar immunoreactivity profiles to those in cartilage extracts (Supplementary Fig. 1; C. M. Milner and A. J. Day, unpublished observation).

Analysis of the relative abundance of bikunin and HCs in CsCl gradient fractions of normal cartilage extracts [Figs. 1(b), 2(b) and 3(b)] and in the final guanidine·HCl extract of the sequential protocol (Fig. 5) revealed the presence of a pool of CS-bound HCs in cartilage which was apparently not associated with bikunin. While the nature of this CSPG is unknown, it is relevant that co-expression of decorin, a major chondrocyte product, with HC3 in COS-1 cells has been shown to generate complexes with HC3 covalently attached to the CS of decorin[18]. Further, characterization of the HCs in human follicular fluid has shown a proportion to be covalently attached to the CS chains of versican[17], which is also synthesized by chondrocytes.

Immunohistochemical analysis for bikunin, HC1 and HC2 of tissue from an individual with entirely normal femoral cartilage (45 y, grade 0) and multiple sites from another with early degenerative cartilage changes (63 y, grade 1) revealed an interesting difference in their distribution. Bikunin-positive cells were only present in cell groups present under fibrillated surfaces in low-grade cartilage, whereas HC-positive cells were found in cell groups under fibrillated surfaces and also under smooth surfaces where there was fibrillation elsewhere in the joint; also a diffuse matrix staining was often seen for HCs but never for bikunin. This is consistent with the idea that the HCs are primarily synthesised by the chondrocytes whereas the bikunin is derived from the synovial fluid. In this regard, synovial fluids from RA[14] and OA[32] patients have been shown to contain HCs linked to HA. Our analysis of CsCl gradient fractions from a pool of synovial fluids derived from OA patients undergoing joint drainage showed the presence of abundant bikunin·CS and also some 90 kDa HCs (but not 50 kDa HCs) linked to HA but not CS. These analyses are consistent with the synovial fluid products being derived from non-cartilage sources and further indicate that the cartilage species are derived primarily from the resident chondrocytes. Further work is needed to describe the details of chondrocyte-mediated biosynthesis of the truncated HCs and the CSPG to which they are complexed. Unravelling these details may provide a means to use specific IαI structures to monitor chondrocyte-specific responses to progressive joint disease.

Conflict of interest

None of the authors have a conflict of interest with respect to the contents of the following paper.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.joca.2008.04.004.

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