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Original Paper

Matrix Metalloproteinase Digestion of Aggrecan in Human Cartilage Tumours

M. Toriyama,[1]* A.E. Rosenberg,[2] H.J. Mankin,[1] T. Fondren,[1] B.V. Treadwell[1] and C.A. Towle[1]

[1]Orthopaedic Research Laboratories GRJ1124; and [2]Department of Pathology, Massachusetts General Hospital 32 Fruit Street, Boston, Massachusetts 02114, U.S.A.

Substantial experimental and clinical evidence suggests that the catabolism of extracellular matrix components is a prerequisite for invasive and metastatic behaviour of solid tumours. Chondrosarcomas are malignant cartilaginous tumours that most commonly arise in bone, and the large aggregating proteoglycan aggrecan is a major component of the extracellular matrix of these tumours. Matrix metalloproteinases (MMPs) have been implicated in tumour invasiveness. The purpose of this study was to determine whether MMPs play a role in aggrecan catabolism in cartilage tumours. In order to detect aggrecan digestion products resulting from *in vivo* cleavage at the MMP site, protein extracts from human articular cartilage and from various cartilage tumours were analysed by Western blot using an antibody to the FVDIPEN neoepitope generated by MMP cleavage. Examination of cartilage extracts revealed a trend of increasing aggrecan digestion at the MMP site with age. One hyaline chondrosarcoma and three osteochondromas lacked detectable aggrecan fragments with the carboxy terminal FVDIPEN neoepitope. Two osteochondromas gave weak signals. However, all chondrosarcomas with degenerating extracellular matrix or with a myxoid component exhibited strong FVDIPEN immunoreactivity. These results demonstrate that, in contrast to the benign cartilage tumour osteochondroma, human chondrosarcomas contain abundant aggrecan degradation products resulting from cleavage *in vivo* at the MMP site in the interglobular domain. These data support the concept that MMPs participate in the degradation of extracellular matrix in chondrosarcoma, allowing the neoplastic chondrocytes to escape local confinement, migrate, and invade neighbouring and remote tissues. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

CHONDROSARCOMAS ARE malignant cartilaginous tumours most commonly arising in bone [1, 2]. According to the study of Dorfman and Czerniak [2], chondrosarcomas represent 25% of all bone sarcomas and are the most frequent primary malignant bone tumour in patients over 50 years of age. Among the common histological variants of chondrosarcoma are the hyaline type, in which the neoplastic chondrocytes reside within well-defined lacunae in an abundant homogeneous safranin O-positive extracellular matrix, and the myxoid type, that contains stellate chondrocytes that are not associated with lacunar spaces and 'float' in a frothy-appearing mucoid matrix. The dedifferentiated version of chondrosarcoma is composed of nodules of low grade hyaline chondrosarcoma that are juxtaposed in a poorly differentiated high grade spindle cell sarcoma that frequently has the appearance of malignant fibrous histiocytoma [1, 3].

Several reports have addressed the proteoglycan-rich extracellular matrix in human chondrosarcomas [3–5], but the enzymes that cleave the large aggregating proteoglycan aggrecan in these tumours have not been characterised.

*Present address: Department of Orthopaedic Surgery, Nihon University School of Medicine, 30-1 Oyaguchi-kamimachi, Itabashi-ku, Tokyo 173, Japan
Correspondence to C.A. Towle.
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Aggrecan is a major component of the extracellular matrix of articular cartilage as well as cartilage tumours. Its degradation during normal and catabolite-stimulated cartilage remodelling appears to be initiated by proteolytic attack within the interglobular domain (Figure 1) [6]. The bond between asparagine and phenylalanine (N_{341}–F_{342}) is susceptible to attack by most members of the matrix metalloproteinase (MMP) family, and degradation products consistent with cleavage at this MMP site accumulate with age in articular cartilage [7–10]. An additional proteolytic target within the interglobular domain of the human aggrecan core protein is the bond between glutamic acid and alanine (E_{373}–A_{374}), which appears to be cleaved by an as yet unidentified enzyme aggrecanase [11]. Recent research has demonstrated that cleavage at E_{373}–A_{374} in the interglobular domain is a crucial step in aggrecan digestion, not only in catabolite-stimulated cartilage explant cultures, but also in osteoarthritic cartilage *in vivo* [11–14].

MMP expression has been found to correlate with malignancy in many types of tumours, and roles for these proteinases in invasion and metastasis have been postulated (reviewed in [15]). We examined the aggrecan digestion products resulting from cleavage at the MMP site in a collection of cartilage tumours by using an antibody to the FVDIPEN neoepitope for Western blot analysis.

MATERIALS AND METHODS

Materials

Chondroitinase ABC and keratanase were purchased from ICN Pharmaceuticals (Costa Mesa, California, U.S.A.). Antipeptide antiserum to FVDIPEN was prepared according to the method of Lark and colleagues [16] and tested for specificity by enzyme-linked immunosorbent assay (ELISA) using a 96-well format with 1 μg peptide per well. Bound antibody was detected using a goat antirabbit IgG–horseradish peroxidase conjugate. Results for each peptide, the immunising peptide FVDIPEN or a truncated variant FVDIPE, were expressed as the reciprocal of the serum dilution that resulted in an OD_{492} of 0.2.

Cartilage and tumour specimens

Articular cartilage was obtained from the ankle joints of patients undergoing amputation for the treatment of tumours at remote sites. The study was reviewed and approved by the Massachusetts General Hospital Human Studies Review Board. Tissue was pulverised in a liquid nitrogen mill and washed in 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM $CaCl_2$, 0.1 mM $ZnCl_2$ and 0.02% NaN_3 (B-50). Powdered cartilage was stored at − 80°C. Tumour specimens obtained at the time of surgery were immediately frozen by liquid nitrogen and stored at − 80°C. Pathological diagnoses were obtained from the patients' medical records, as were details of the patient's age and sex, tumour location, ploidic distribution, and pre-operative therapy. Histological slides prepared from frozen tissue samples, more closely reflecting the tissue analysed by Western blot, were examined to determine whether the histopathology was consistent with the recorded diagnoses. Safranin-O stained slides were examined to evaluate the proteoglycan content.

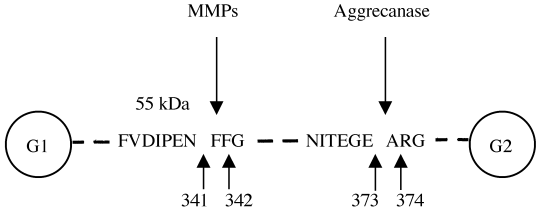

Figure 1. Diagram of the human aggrecan interglobular domain. The aggrecan polypeptides produced by cleavage at the preferred site for matrix metalloproteinases (MMPs) (N_{341}–F_{342}) and aggrecanase (E_{373}–A_{374}) are illustrated. Cleavage at the MMP site produces a 55 kDa G1 containing fragment with the FVDIPEN C-terminus.

Preparation of tissue extracts

Powdered human articular cartilage (10 mg) was resuspended in 2.5 ml B-50 and collected by centrifugation. Chondrosarcoma tissues (approximately 100 mg) were washed with 10 ml phosphate buffered saline (PBS) containing 10 mM ethylene diamine tetraacetic acid (EDTA), 1 mM phenyl methyl sulphonyl fluoride (PMSF) for 1 h at 4°C. Two millilitres of 4 M guanidine HCl, 20 mM EDTA, 1 mM PMSF was added to each powder or tissue sample, and the samples stirred in a cold room overnight. Extracts were dialysed against 0.1 M Na acetate, 0.1 M Tris–HCl, pH 6, 1 mM EDTA for 24 h at 4°C.

Estimation of proteoglycan content

The sulphated glycosaminoglycan content of extracts was estimated spectrophotometrically using the metachromatic dye 1,9-dimethyl methylene blue [17]. Proteoglycan content of crude extracts, expressed as chondroitin sulphate equivalents per wet weight of tissue, was derived (without correction for keratan sulphate or other components contributing to the change in absorbance) from a calibration curve created using whale chondroitin sulphate as a standard.

Preparation of samples for electrophoresis

Chondroitinase (0.002 units/100 μg chondroitin sulphate) was added to each dialysed extract, and the extracts were digested overnight at 37°C. Keratanase (0.005 units/100 μg chondroitin sulphate) was added and the incubation continued for 2 h. Deglycosylated extracts were adjusted to 0.5 M NaCl, 0.1 M Na acetate, 0.1 M Tris–HCl, pH 6 and passed over a 6 ml DEAE Sephadex A-50 column equilibrated in the same buffer. Flow through material was concentrated to dryness using an Amicon (Beverley, Massachusetts, U.S.A.) concentrator. Concentrated material was dissolved in B-50, and the protein was precipitated with two volumes of acetone. Each sample was dissolved in B-50 at 10 mg/ml based on the initial chondroitin sulphate determination.

Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis

Samples equalised for proteoglycan (100 μg chondroitin sulphate prior to deglycosylation) were adjusted to 1% SDS, 5% glycerol, 1% 2-mercaptoethanol and resolved on SDS polyacrylamide (12%) gels.

Western blotting to detect FVDIPEN neoepitope

Gels were equilibrated with 10 mM 3-(cyclohexylamino)-1-propane sulphonic acid, pH 11, 5% methanol, and the resolved proteins were electrophoretically transferred to Immobilon. Membranes were blocked by incubation in 3% gelatin in 20 mM Tris–HCl, pH 7.8, 150 mM NaCl, 0.02% NaN_3 (TBS) and then incubated overnight with anti-FVDIPEN antiserum diluted 1:2000 in 1% gelatin in TBS. As a

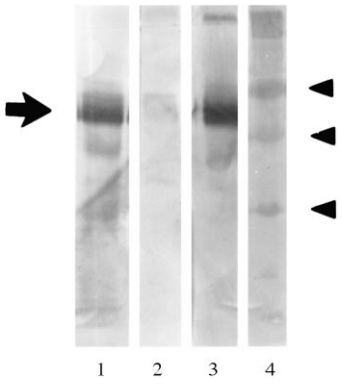

Figure 2. Western analysis demonstrated the neoepitope specificity of the antiserum. The 55 kDa signal was abrogated by pre-incubation of the antiserum with FVDIPEN (lane 2), but not with FVDIPE (lane 3), as compared with the control antiserum (lane 1). Lane 4, standards. Arrowheads indicate standards of 69, 46, and 30 kDa.

Figure 3. FVDIPEN immunoreactivity in human articular cartilage. An age-dependent increase in anti-FVDIPEN reactive polypeptide was apparent in human articular cartilage (lane 1, 15 year old; lane 2, 37 year old; lane 3, 47 year old).

control for specificity of the antiserum, two additional blots were prepared in parallel for staining with the antiserum pre-incubated with 50 μg of the immunising peptide (FVDIPEN) or with a truncated version of the peptide (FVDIPE). Blots were washed with TBS containing 0.05% Tween-20 (TBST) and incubated for 1 h with 1:3000 dilution of alkaline phosphatase conjugated goat antirabbit IgG (Biorad, Hercules, California, U.S.A.). The blots were washed with TBST and developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the chromogenic substrate.

RESULTS

Characterisation of the antineoepitope antiserum

The titre by ELISA of the anti-FVDIPEN antiserum was 16 400 against the immunising peptide FVDIPEN as compared with 300 against the truncated peptide FVDIPE. Extracts of articular cartilage or cartilage tumours were analysed by Western blot for reactivity with this antiserum. In all FVDIPEN positive samples, the predominant immunoreactive species migrated as a 55 kDa polypeptide, the size expected for the entire G1 domain terminating at N_{341}. No immunoreactivity was evident in the region of the gel corresponding to aggrecan core protein (370 kDa), demonstrating that the antiserum recognises the 'neoepitope' or new carboxy terminus created by cleavage of aggrecan core protein at the MMP site, N_{341}–F_{342}. The 55 kDa signal was abrogated if the antipeptide antiserum was preincubated with the immunising peptide (FVDIPEN), but not following pre-incubation with a truncated version (FVDIPE) (Figure 2).

FVDIPEN immunoreactivity in human articular cartilage

Normal cartilage from a 15 year old patient did not contain detectable aggrecan degradation products with the C-terminal FVDIPEN neoepitope. Cartilage from a 37 year old was weakly positive, and cartilage from a 47 year old patient gave a strong signal on the Western blot at 55 kDa (Figure 3).

Table 1. *Clinical data and characterisation of tumour specimens*

	Age/sex	Pathology	Location	Ploidy	Pre-operative	Saf-O	FVDIPEN	CS (μg/100 mg)
1	48/M	O-CH (with CHSA)	Femur	A	–	+	–	1000
2	34/M	O-CH	Femur	D	–	+	–	2933
3	43/M	O-CH	Femur	D	–	+	–	2680
4	46/M	O-CH	Fibula	D	–	+	+/–	977
5	41/M	O-CH	Femur	D	–	+	+/–	988
6	33/M	G1 (H)	Pelvis	D	–	+	–	1078
7	37/F	G1 (H,M)	Pubis	A	–	–	+++	228
8	67/M	G2 (H,M)	Tibia	D	–	–	++	287
9	63/F	G1–2 (H,M)	Pelvis	A	RT	–	++	728
10	36/M	G1–2 (H,M)	Spine	A	CT, RT	–	+++	206
11	37/M	G2 (H,M)	Humerus	?	–	–	++	167
12	71/M	G2–3 (DE)	Ilium	A	–	–	+++	643

Pathological diagnoses were obtained from medical records and confirmed by examination of slides from the sampled tissue specimens. O-CH, osteochondroma; CHSA, chondrosarcoma; H, hyaline type chondrosarcoma; H,M, mixed hyaline and myxoid type chondrosarcoma; DE, dedifferentiated chondrosarcoma and grade (G1 to G3). With one exception, histopathology of the tissue specimen was consistent with the recorded diagnosis. The diagnosis in the medical record for patient no. 1 was osteochondroma with chondrosarcoma. However, the tissue sample for this study was evaluated as osteochondroma. Ploidy was determined by flow cytometry of tumour cell suspensions stained with propidium iodide: D, diploid; A, aneuploid. 2 patients received pre-operative treatment: RT, radiation; CT, chemotherapy. Chondroitin sulphate (CS; μg/100 mg wet weight), sulphated glycosaminoglycan in crude extracts was estimated by metachromatic dimethyl methylene blue using whale chondroitin sulphate to generate the calibration curve. Paraffin sections of frozen tumour specimens were stained with safranin-O to visualise proteoglycan. +, intense safranin-O staining of cartilaginous matrix. –, safranin-O staining weak or limited to small portion of tissue; FVDIPEN immunoreactivity was rated as undetectable (–), weak (+/–), strong (++), or very strong (+++).

FVDIPEN immunoreactivity in human osteochondroma and chondrosarcoma

All tumour extracts contained sulphated glycosaminoglycan as determined by the metachromatic dye assay, with osteochondromas and one hyaline type chondrosarcoma higher in proteoglycan content (977–2933 μg chondroitin sulphate equivalent per 100 mg) than the other chondrosarcomas (167–728 μg/100 mg; Table 1). Extracts from three osteochondroma specimens and one hyaline type chondrosarcoma were negative for FVDIPEN staining, and two cases of osteochondroma produced a relatively weak signal. Histological sections of these specimens stained strongly by safranin-O, consistent with the biochemical assay and indicative of abundant proteoglycan. Six specimens of chondrosarcoma with a myxoid component and/or with extracellular matrix depleted of proteoglycan based on weak safranin-O staining exhibited strong FVDIPEN immunoreactivity (Figure 4 and Table 1).

DISCUSSION

A major component of the abundant extracellular matrix of articular cartilage is the large hyaluronan-binding proteoglycan aggrecan. The carboxy terminal portion of aggrecan is heavily substituted with chondroitin and keratan sulphate and enables the cartilage to resist compression under load. Aggrecan catabolism during normal and pathological cartilage remodelling involves proteolytic cleavage within the interglobular domain, releasing chondroitin and keratan sulphate-rich fragments from the hyaluronan-binding globular domain, G1 (Figure 1).

As in articular cartilage, the extracellular matrix of cartilage tumours contains aggrecan. However, no studies have been published on the catabolism of this proteoglycan in human chondrosarcoma, a malignant neoplasm of cartilage. The issue is an important one because destruction of the proteoglycan-rich extracellular matrix in cartilage tumours is likely to be a prerequisite for invasive and metastatic behaviour. MMPs have been implicated in tumour invasiveness [15], and MMP mRNAs have been detected in cartilage tumour specimens [18]. However, it is unknown whether MMPs are proteolytically active in these tumours *in vivo*.

To detect aggrecan fragments resulting from cleavage *in vivo* at the MMP site, extracts of human articular cartilage, osteochondroma (a benign cartilage tumour) and chondrosarcoma were examined for the presence of carboxy terminal FVDIPEN neoepitope by Western blot analysis. In the articular cartilage specimens, an age-dependent increase in anti-FVDIPEN reactive 55 kDa polypeptide was apparent.

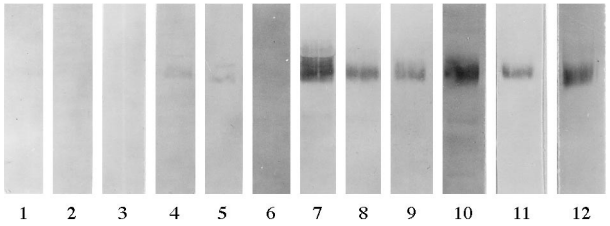

Figure 4. FVDIPEN immunoreactivity in benign and malignant cartilage tumours. Osteochondroma (lanes 1–5) and one hyaline type chondrosarcoma with high proteoglycan content (lane 6) had minimal FVDIPEN positive aggrecan catabolites. Chondrosarcomas with myxoid component and/or low proteoglycan content (lanes 7–12) exhibited strong immunoreactivity.

This result shows that cleavage at the MMP site is one step in the catabolic destruction of aggrecan in the cartilage of older individuals, consistent with a previous report demonstrating the accumulation of anti-FVDIPEN positive aggrecan degradation products in cartilage with age [7].

The benign cartilage tumours (osteochondromas) and one hyaline type chondrosarcoma showed little or no evidence of aggrecan cleavage between N_{341} and F_{342}. These tumours stained well by safranin-O, indicative of abundant proteoglycan in the extracellular matrix. However, all human chondrosarcomas having a myxoid component and all chondrosarcomas with degenerating extracellular matrix, as evidenced by weak safranin-O staining, produced a positive signal for 55 kDa immunoreactivity. This result is evidence that the chondrosarcoma aggrecan is degraded by cleavage at N_{341}–F_{342}, most likely by one or more of the well-characterised MMPs [7–10].

In diseases such as osteoarthritis and rheumatoid arthritis, where proteolysis results in the loss of cartilage function, cleavage between glutamic acid and alanine (E_{373}–A_{374}) by an as yet unidentified enzyme aggrecanase may be critical to aggrecan catabolism [11–14]. The possibility that aggrecanase is active in chondrosarcoma, perhaps cleaving at E_{373}–A_{374} in the aggrecan interglobular domain as a prerequisite to MMP cleavage, is not addressed by our study. The MMP site (N_{341}–F_{342}) lies between the hyaluronan-binding domain G1 and the aggrecanase site at E_{373}–A_{374} (Figure 1). Thus, the presence of hyaluronan-binding anti-FVDIPEN reactive G1 fragments in chondrosarcoma tissue does not preclude the possibility that aggrecanase is important, possibly the earliest acting enzyme in aggrecan catabolism. Our attempts to identify anti-NITEGE neoepitopes resulting from cleavage at E_{373}–A_{374} in human chondrosarcoma were unsuccessful. However, our antiserum to this neoepitope, which is capable of detecting G1 fragments in extracts of adult articular cartilage, is neither as specific nor as sensitive as the anti-FVDIPEN reagent. A more thorough analysis of aggrecan catabolites is required to define the critical or rate-limiting enzyme(s) in chondrosarcoma.

Mankin and colleagues [3] reported that type I chondrosarcomas, hyaline cartilage tumours with high hexoseamine content (a measure of proteoglycan), were the least malignant among a large collection of cartilage tumours, while type II chondrosarcomas (hyaline with low hexoseamine content) were slightly more malignant. In that same study, myxoid and dedifferentiated tumours were most frequently high grade chondrosarcomas. Huvos [1] suggested that the presence of myxomatous change and cystic degeneration in human chondrosarcoma correlates well with low and medium grade malignancy.

Under certain pathological conditions, there is believed to be a proteolytic imbalance between enzyme(s) and endogenous inhibitor(s), allowing the neoplastic cells to invade surrounding tissue and metastasise [19]. The results of the study presented here show that cleavage of aggrecan at the MMP site occurs *in vivo* in human chondrosarcomas, suggesting the possibility that an imbalance between the MMPs and their physiological regulators, the TIMPs (tissue inhibitor of metalloproteinase), contributes to the invasive and metastatic potential.

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