Osteoarthritis and Cartilage (2010) **18,** 397–405

doi:10.1016/j.joca.2009.09.011

Osteoarthritis and Cartilage

Collagen fibril disruption occurs early in primary guinea pig knee osteoarthritis

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Summary

Objective: A major barrier inhibiting the discovery of structural modifying agents for osteoarthritis (OA) is an incomplete understanding of early disease events. Herein, we investigated the time course of collagen II cleavage and fibril disruption in the well-validated Hartley guinea pig model of spontaneous OA of the knee.

Methods: Knee joints of 46 male Hartley guinea pigs were analyzed at 3 weeks, 2, 4, 7, 10, 12, and 18 months of age for histological severity of OA, cartilage collagen fibril disruption by semi-quantitative polarized light microscopy, and expression of type II collagen degradation biomarkers, 9A4 and Coll2-1, by immunohistochemistry. In addition, serum biomarkers specific for collagen II degradation, CTX-II, C2C, and Coll2-1 were quantified.

Results: Collagen fibril disruption and expression of the collagenase-generated cleavage neoepitope, 9A4, were observed as early as 2 months of age, despite the appearance of histological OA at 4 months of age. Only serum Coll2-1 increased coincident with the early disruption of the collagen fibril between 3 weeks and 7 months, in contrast to serum C2C, which did not change significantly or correlate with histological severity. Inversely, CTX-II declined dramatically from 3 weeks to 4 months and remaining low thereafter, coincident with growth plate turnover.

Conclusions: Collagenase cleavage and disruption of the type II collagen network are early OA disease events in this model, preceding histological evidence of proteoglycan loss. The markedly different serum profiles of collagen II-related biomarkers during the early stages of disease development suggest compartmental segregation and temporal regulation of collagen degrading enzymes.

Key words: Osteoarthritis, Collagen, Biomarkers, Animal model, Guinea pig.

Introduction

A greater focus on early, pre-radiographic osteoarthritis (OA) is needed to provide insight into targets for intervention at a stage more amenable to modification. The early events are difficult to characterize in primary human OA due to the great uncertainty surrounding disease onset and the great biological variability in disease progression. The Hartley guinea pig however provides a consistent model of spontaneous and progressive degeneration of the knee that closely resembles knee OA in humans[1,2]. This animal model is especially attractive for studies of OA pathology because it represents primary idiopathic OA, and the guinea pig, unlike rodents, expresses both interstitial collagenases-1 and -3 which are associated with cartilage damage[3], and OA in humans[4]. We hypothesized that the Hartley guinea pig would provide a physiologically relevant model in which to analyze early OA disease events to gain insights into mediators of disease onset. This study focused on characterizing the collagen II-related events relative to histological OA and proteoglycan loss in the course of disease development.

The collagen fibril network maintains the volume, shape, and tensile strength of the extracellular matrix of articular cartilage. Type II collagen is relatively specific to articular cartilage and is the most abundant cartilage protein, representing 15–25% of the wet weight, 50% of the dry weight[5], and >90% of the total collagen content[6]. Disruption of this fibrillar network is mediated by specific matrix metalloproteinase (MMP) enzymes that cleave collagen, namely collagenases-1, -2 and -3 (MMPs-1, -8, and -13). Collagenase-mediated cleavage of type II collagen produces two fragments: a 3/4 length fragment and a ¼ length fragment. Once this initial cleavage has taken place, the triple helical collagen fibril unwinds and becomes susceptible to degradation by other enzymes such as gelatinases. The biological half-life of type II collagen is estimated to be 117 years in human adult cartilage[7], contributing to the concept that collagen fibril degradation is a key feature of OA because the irreversible damage imposed on this matrix molecule is not adequately compensated for by new protein synthesis.

The proteolysis of type II collagen yields three types of neoepitope fragments accessible to body fluids[8]: the collagenase-generated C2C neoepitope on the carboxy end of

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Received 14 May 2009; revision accepted 27 September 2009.

the 3/4 fragment[9], the denaturation-generated Coll2-1 neoepitope[10] in the triple helical domain near the carboxy end of the 3/4 fragment, and the matrix metalloprotease-generated neoepitope[11,12] CTX-II[13], localized to the mature carboxy end or telopeptide of the collagen molecule (Fig. 1). We have found that all three types of collagen II neoepitopes are measurable in the guinea pig, providing a means of quantifying the time course of collagen II metabolism in this animal model. These provided adjunctive measures with which to monitor the disease course to complement the primary histological, and polarized light microscopic outcomes of collagen degradation in this model system.

Methods

ANIMALS

Six male Hartley guinea pigs were obtained from Charles River Laboratories (Wilmington, MA) at 3 weeks of age, and an additional forty male Hartley guinea pigs were obtained at 2 months of age and raised until sacrifice at 2 ($n=6$), 4 ($n=6$), 7 ($n=6$), 10 ($n=6$), 12 ($n=6$), and 18 ($n=10$) months of age. The Institutional Animal Care and Use Committee approved all procedures. Blood was collected into SST Vacutainer tubes (VWR International, West Chester, PA) at each time point. The samples were centrifuged at 3000 rpm for 15 min and sera were aliquoted and frozen at −80 °C until analyzed. The right knee joint from each animal was fixed for 24 h in 10% buffered formalin, followed by decalcification in 10% ethylenediaminetetraacetic acid (EDTA) in 0.1 M phosphate buffer, pH 7.6–7.8. Paraffin sections (5 μM) of the central region of the joint were stained with either toluidine blue for histological analyses or picrosirius red and examined under polarized light to evaluate collagen birefringence.

HISTOLOGICAL GRADING

A semi-quantitative modified Mankin histological grading system described previously[14] was used to evaluate OA severity in each tibial plateau and femoral condyle. This scoring scheme permits site-specific and separate scoring of cartilage structure (extent and severity of surface irregularities including fibrillation and clefts [0–8]), and proteoglycan content (as determined by extent of toluidine blue staining [0–6]) and has demonstrated a correlation with severity of OA as reflected by the synovial fluid biomarkers, cartilage oligomeric matrix protein (COMP) and keratan sulfate (KS)[14–16]. Histological sections were graded by two observers (JLH, VBK) blinded to the animal status.

POLARIZED LIGHT MICROSCOPIC ANALYSES

Slides containing histological sections for analyses of collagen network orientation were exhaustively deparaffinized, as paraffin is strongly birefringent. Sections were hydrated as described previously[17] and treated at 37 °C for 18 h in 2.0 mg bovine testicular hyaluronidase in 1.0 ml 0.1 M phosphate buffer at pH 6.0 to remove chondroitin sulfate molecules[18], to eliminate possible masking of collagen's cationic binding sites for the polyanionic

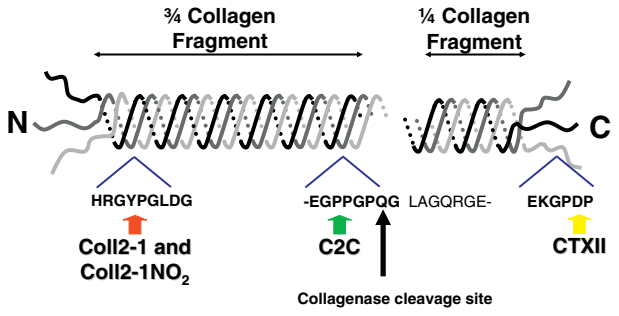

Fig. 1. Schematic representation of the specific type II collagen epitopes measured. The epitopes measured in sera result from the three types of collagen degradation: collagenase cleavage (C2C), denaturation (Coll2-1), and MMP-generated collagen telopeptides (CTX-II).

Sirius red molecules[19]. Sections were then stained for 30 min in 0.1% Sirius red F3B (Polysciences, USA) dissolved in saturated picric acid, which enhances the normal birefringency of collagen fibers in tissue sections[20], and then washed, dehydrated, and mounted with cover slips. Sections were analyzed with a Nikon microscope equipped with polarizing filters and the induced birefringence was determined by turning the analyzer in two opposite directions. The optical properties of the extracellular matrix, namely the presence or absence of birefringence, indicated the orientation of the collagen fibers[20,21] of the articular cartilage. The relative loss of collagen birefringence (expressed as a percentage) was based on a comparison with the pattern of birefringence in the 3-week old guinea pig knee cartilage which was taken to represent normal (100%) birefringence. These assessments were made blinded to animal age and histological score. The images were obtained using Metamorph Software 4.01™. The entire area of articular cartilage was circumscribed to obtain a total area. Likewise, areas of normal vs altered birefringence were circumscribed allowing expression of birefringence results as percentages of total cartilage area.

IMMUNOHISTOCHEMISTRY

Sections of guinea pig knees were stained with monoclonal antibody (mAb) 9A4 (kindly provided by Dr. Peter Mitchell) and antisera D3 containing antibodies to Coll2-1. The 9A4 mAb recognizes the collagenase-generated neoepitope at the C-terminus of the collagen 3/4 fragment[22] and was readily available whereas the anti-C2C antibody was not commercially available and has been reported to be unsuitable for immunohistochemistry[23]. The antiserum to Coll2-1 recognizes a denaturation epitope of collagen II that results from unwinding and thus detects only degraded collagen, ether *in situ* (in cartilage) or in body fluids[24]. Paraffin-embedded sections were deparaffinized with xylene and then rehydrated with graded ethanol. Endogenous peroxidase activity was blocked by incubation of the sections with freshly prepared 0.5% (vol/vol) H_2O_2 in absolute ethanol for 10 min at room temperature. To enhance the permeability of the extracellular matrix, glycosaminoglycans were removed by incubating the sections with 0.4 U/l proteinase-free chondroitinase ABC (Sigma) in 0.1 M Tris–HCl pH 8.0 for 30 min at 37 °C. Non-specific binding was blocked by incubation of the sections with 100 μl of 1% normal goat serum (Jackson ImmunoResearch) diluted in tris buffered saline (TBS); 50 mM Tris, 138 mM NaCl pH 7.6 for 30 min. For staining with mAb 9A4, sections were incubated overnight at 4 °C with 15 μg/ml of mAb 9A4 according to previously described methods[3]. Staining for Coll2-1 was performed by incubating sections for 2 h with 100 μl of antisera derived from rabbits (D3) diluted 1/100 in TBS containing 1% normal goat serum, as described previously[23]. As a negative control, sections were treated with goat non-immune serum diluted 1/100 as the substitute for the primary antibody.

COLLAGEN II BIOMARKER ANALYSES

Levels of serum C2C were quantified by a commercially available enzyme-linked immunosorbent assay (ELISA) (IBEX, Montreal, Quebec) in serum diluted 1:2, as per the manufacturers' protocol. The intra- and inter-assay variability for C2C was 3.3% and 16.3% respectively, as reported previously[16]. The denaturation epitope, Coll2-1 and the nitrosylated epitope, Coll2-1NO_2, localized to the helical domain of type II collagen[24,25] were quantified in guinea pig sera (diluted 8-fold) by competitive ELISA in duplicate with polyclonal rabbit antisera (D3 and D37, respectively)[24]. For Coll2-1, the intra-assay and inter-assay variability were 8.2% and 9.3% respectively. Levels of serum CTX-II were quantified using the serum Preclinical Cartilaps ELISA (Nordic Bioscience), an assay designed to detect degradation products of C-terminal telopeptides of type II collagen in animal sera. The intra-assay and inter-assay variability were 5.1% and 8.9% respectively. Biomarker assays were performed blinded to guinea pig age or histological data. Results were expressed as mean + standard deviation (SD).

STATISTICAL ANALYSES

All statistical analyses were performed using Prism GraphPad 5.0 (GraphPad Software, La Jolla, CA). The individual components of the histological scores (cartilage structure and proteoglycan content) and the birefringence of the four joint surfaces [medial and lateral femoral (LF) condyles, medial and lateral tibial (LT) plateaus] at the various ages were analyzed by the one-way analysis of variance (ANOVA), non-parametric Kruskal–Wallis test, followed by Dunn's Multiple Comparison post-hoc test; the non-parametric Mann–Whitney test was used to compare the differences in histological scores and measures of birefringence between either medial or lateral compartments or tibial and femoral surfaces, as well as the levels of Coll2-1 at 3 weeks and 4 months of age. To evaluate for potential correlation of the biomarkers, non-parametric Spearman correlations were calculated. In addition, total histological scores were separated into quartiles and corresponding levels of biomarkers were analyzed by one-way ANOVA, non-parametric Kruskal–Wallis