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Detection of collagen type II and proteoglycans in the synovial fluids of patients diagnosed with non-infectious knee joint synovitis indicates early damage to the articular cartilage matrix

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Summary

Objective: We have sought to determine if markers of proteoglycans and collagen type II (CII) degradation can be detected at an early stage following acute knee injury in the synovial fluid (SF) from a group of patients diagnosed with non-infectious knee joint synovitis (KJS). CII, proteoglycans and elastase activity in the SF from patients with KJS were compared to SF from patients with two chronic arthritis conditions: osteoarthritis (OA) and rheumatoid arthritis (RA) as well as normal SF controls.

Methods: CII peptides were measured by sandwich ELISA using two monoclonal antibodies: 8:6:D8, a CII-specific antibody, and 14:7:D8 which binds to an amino acid sequence on CII as well as collagens type I, III and V. Epitope 9A4, a neo-epitope resulting from collagenase digestion of CI, CII, and CIII was measured by inhibition ELISA. Proteoglycans measurement included total sulfated glycosaminoglycans (sGAG) by dye-binding assay and 5-D-4 epitope, a keratan sulfate epitope, by inhibition ELISA. Elastase activity was measured colorimetircally using *N*-succinyl trialanine *p*-nitroanilide (SANA) substrate.

Results: The quantified CII peptide concentrations by sandwich and inhibition ELISA were significantly higher in SF from patients with KJS ($P<0.05$) compared to SF from patients with OA, RA and normal aspirates. 5-D-4 and sGAG concentrations were significantly lower ($P<0.05$) in SF from patients with KJS compared to SF from patients with OA and RA. Elastase activity in SF from patients with KJS and RA were significantly higher ($P<0.05$) than SF from patients with OA. A significant correlation exists between elastase activity and 9A4 epitope concentration in SF from patients with KJS.

Conclusion: The elevated CII peptides concentrations in KJS SF compared to normal and OA aspirates indicate early signs of cartilage network damage. The low proteoglycans concentrations in SF from patients with KJS may indicate that injury is limited to the superficial zone of cartilage in the patient population studied. The high elastase activity in SF from patients with KJS and RA are linked to the high CII peptides concentration. The elastase activity in the SF from patients with KJS is due to the action of neutrophil elastase (NE) and collagenases, where both contribute to the destruction of the articular cartilage.

Key words: Synovitis, Cartilage, Osteoarthritis, Collagen type II.

Introduction

Chronic arthritis conditions, osteoarthritis (OA) and rheumatoid arthritis (RA), are characterized by irreversible damage to the cartilage matrix caused by enzymatic degradation of the two major components of cartilage: collagen type II (CII) and aggrecan[1–4]. As a result of this breakdown, molecular markers of aggrecan and CII degradation appear in the synovial fluids (SF) of the affected joints. Sulfated glycosaminoglycans (sGAG) in SF indicate the extent of aggrecan degradation[4]. Monoclonal antibodies against CII molecule have been developed and utilized in ELISA immunoassays to measure CII fragments in the SF[5–7].

Acute knee injury either due to injury or repetitive impact loading is recognized as an apparent and distinct preventable cause of secondary OA[8]. The mechanism by which secondary OA develops after acute knee injury is still unclear. Evidence of cartilage damage, if present, at an early stage following knee injury can help explain the biochemical processes that can lead to the late development of secondary OA. This study sought to examine the presence of molecular markers of CII and proteoglycans degradation in the SF from a patient population diagnosed with non-infectious knee joint synovitis (KJS) at an early stage following a knee injury.

CII and proteoglycans SF concentrations of patients with KJS were compared to those of two patient groups with chronic arthritis conditions: OA and RA. The elastase activity among the three patient groups was compared and potential correlations between elastase activity in SF from patients with KJS and cartilage matrix degradation were investigated. A novel sandwich ELISA was developed for this investigation using 18:6:D6, a CII-specific antibody that reacts with epitopes on cyanogen bromide (CNBr) cleaved

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peptides CB 9.7 and CB 11[9], and 14:7:D8 which reacts with an identical amino acid sequence in CII as well as collagens type I, III and V[9]. The C-terminal neo-epitope derived from collagenase degradation of fibrillar collagen was quantified using monoclonal antibody 9A4[6]. Measurement of proteoglycan degradation included sGAG and 5-D-4 SF concentrations.

Patients and methods

STUDY DESIGN

This was an observational study of CII and proteoglycans degradation markers in aspirated SF from an emergency department patient population presenting with an acute knee injury as compared to SF from patients with advanced arthritis conditions and normal SF aspirates.

STUDY SETTING AND PATIENT SELECTION

Patients diagnosed with KJS (48 patients) were enrolled in the study. Institutional review board (IRB) approvals were obtained prior to the initiation of the study. Aliquots of SF were taken from both pediatric and adult emergency department patients undergoing diagnostic knee joint arthrocentesis. Inclusion criteria for patients with KJS included monoarticular knee joint effusions judged to be clinically significant, requiring arthrocentesis in the absence of radiographic evidence of fracture. Exclusion criteria included a diagnosis of occult fracture, history of sickle cell anemia or hemophilia, patellar bursitis, gout, pseudogout, and samples contaminated with blood.

An 18-gauge needle was used, under aseptic techniques, to aspirate SF from the lateral aspect of the affected knee joint following anesthesia of the site with 2% lidocaine. Aspirations were performed by both emergency medicine attendings and resident physicians. Informed consent was not obtained, as these were samples, which would otherwise be discarded.

Aspirates were also obtained from patients diagnosed with a Noyes criteria grade III or IV OA, 72 patients, and patients diagnosed with RA, 20 patients. These were obtained during arthroscopy and rheumatologic evaluation, respectively. Normal human SF was obtained from human subjects postmortem who showed no signs of gross degenerative joint disease following fatal accidents. These samples were obtained *in gratis* from Dr Martin Lotz at Scripps Research Institute.

HYALURONIDASE SF TREATMENT

SF samples were collected, aliquoted and stored at –70°C. Sample aliquots used in the elastase activity assays were not treated with EDTA. Other aliquots were treated with 5 mM EDTA and centrifuged at 10 000 rpm for 15 min to remove debris. SF samples were treated with streptomyces hyaluronidase (Sigma–Aldrich, Saint Louis, MO, USA) to a final concentration of 200 U/ml for 40 min at 56°C and subsequently for 20 min at 80°C.

SANDWICH ELISA FOR CII PEPTIDES

Two CII monoclonal antibodies, 18:6:D6 and 14:7:D8, were used in this assay[9]. Antibody 18:6:D6 was made against CII CNBr cleavage peptide 9.7 coupled to ovalbumin. Antibody 14:7:D8 was made using a 15 amino acid synthetic peptide *GPQGPRGDKGEAGEP* coupled to KLH and was conjugated to horseradish peroxidase by using EZ-Link maleimide activated horseradish peroxidase kit (Pierce Chemicals, Rockford, IL, USA). Microtiter plates (High binding Costar, Corning Inc., Corning, NY, USA) were coated with a 1:500 dilution of 18:6:D6 antibody in phosphate buffer saline (PBS) overnight. Each well received 100 µl of the antibody solution. Hyaluronidase-pre-treated SF samples (serial dilutions in PBS–Tween 20), CNBr cleaved CII peptides spiked SF samples and standards (different dilutions of CNBr cleaved CII peptides) were incubated on the pre-coated plates for 60 min at room temperature. Antibody 14:7:D8 conjugated with peroxidase in PBS–Tween, a 1:1000 dilution, was subsequently added for 60 min at room temperature. Fluorescence substrate developing solution (100 µl) (QuantaBlu™, Pierce) was added for 60 min and the fluorescence was measured at an excitation wavelength 330 nm and an emission wavelength 460 nm using a Packard Fluorocounter (Packard Instruments, Meriden, CT, USA).

TOTAL PROTEIN LEVELS

Total protein concentrations of SF samples were determined colorimetrically using Micro BCA™ Protein Assay Reagent Kit (Pierce). A series of bovine serum albumin standards, 100 µl, (100–2 µg/ml) as well as SF samples diluted in distilled water, 100 µl, were incubated with Micro BCA reagent, 100 µl in the wells of a 96-well plate for 60 min at 37°C. The absorbance was subsequently measured at 570 nm using a Dynex MRX microtiter plate reader (Dynex Technologies, Chantilly, VA, USA).

DETERMINATION OF HYALURONATE CONCENTRATION

SF samples were assayed by the manual carbazole reaction for uronic acid[10]. SF samples, diluted in PBS (125 µl) were mixed with concentrated H_2SO_4 containing 25 mM sodium borate previously cooled to –70°C (750 µl). The mixture was heated at 100°C for 10 min and subsequently cooled to room temperature. Carbazole solution in absolute ethanol, 0.125% (w/v) was subsequently added to the reaction mixture (25 µl). After heating at 100°C for 15 min, the presence of hyaluronate was indicated by a pink color that was measured spectrophotometrically at 530 nm.

INHIBITION ELISA FOR 9A4 EPITOPE

Antibody 9A4 was kindly provided by Dr Ivan Otterness, Pfizer Central Research, Groton, CT. A proline rich, nine amino acid peptide *AEGPPGPQG* to which 9A4 has shown a high binding affinity[11], was synthesized using fmoc chemistry and used to coat microtiter plates at a final concentration of 500 ng/ml in 50 mM sodium carbonate buffer, pH 9.5 overnight at 4°C. The nine amino acid peptide in serial dilutions (2–0.001 µg/ml) and hyaluronidase-pre-treated SF samples were incubated with equal volumes of 9A4 antibody (1:1000) dilution in PBS–Tween overnight at room temperature. Samples of the standards or the SF treated with 9A4 antibody (100 µl) were incubated on the pre-coated microtiter plates for 60 min at room temperature. Goat anti-mouse IgG (H+L)-HRP conjugate (Bio Rad, Hercules, CA, USA), 1:1000 dilution in PBS–Tween was subsequently added for 60 min at room temperature. The assay was developed using a fluorescence substrate in a manner identical to the CII sandwich ELISA.

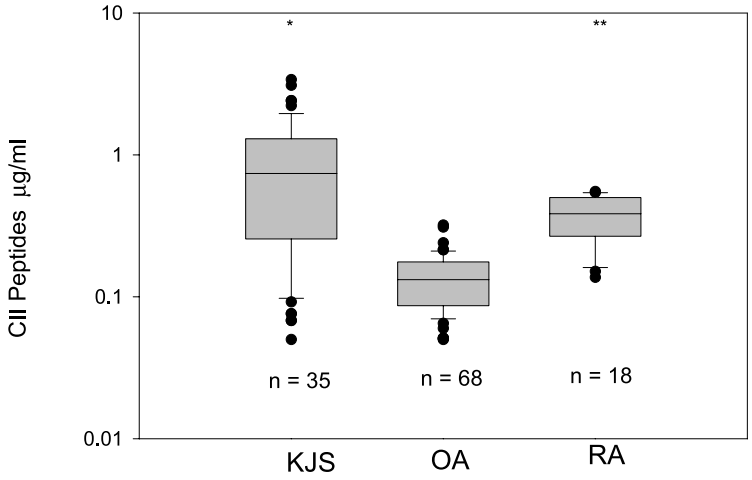

Fig. 1. Collagen type II (CII) peptides concentrations in SF from patients with KJS, OA and RA using a sandwich ELISA. * CII peptides concentrations in KJS were significantly higher (P<0.001) than both OA and RA aspirates. ** CII peptides concentrations in RA were significantly higher (P<0.001) than OA aspirates.

INHIBITION ELISA FOR 5-D-4 EPITOPE

Microtiter plates were coated with chondroitinase-ABC treated[12] bovine aggrecan monomer A_1D_1 to a final concentration of 1 µg/ml in 50 mM sodium carbonate buffer, pH 9.5 overnight at 4°C. Standard bovine aggrecan A_1D_1 monomer in serial concentrations (4–0.03 µg/ml) and hyaluronidase-treated SF samples were incubated with equal volumes of 5-D-4 antibody (ICN Biomedicals Inc., Irvine, CA, USA), 1:10 000 dilution in PBS–Tween, overnight at room temperature. Samples of the standards or the SF treated with 5-D-4 antibody (100 µl) were incubated on the pre-coated microtiter plates for 60 min at room temperature. The assay was further developed in a method similar to the ELISA for 9A4 epitope.

DETERMINATION OF TOTAL SGAG

Total sGAG levels were determined by the 1,9-dimethylmethylene blue (DMB; Sigma–Aldrich) dye-binding assay[13]. Bovine tracheal chondroitin sulfate (Sigma–Aldrich) was serially diluted in distilled deionized water as a standard and the shift in absorbance was measured immediately after adding the dye solution at 570 nm.

ELASTASE ACTIVITY IN SF

Hydrolysis of a low molecular weight synthetic substrate *N*-succinyl trialanine *p*-nitroanilide (SANA, Sigma–Aldrich), which releases *p*-nitroaniline, was measured spectrophotometrically at 410 nm[14] and used as a measure of SF elastase activity. Pancreatic porcine elastase type I (6.8 U/mg, Sigma–Aldrich) was used to construct a standard curve. One unit of the enzyme was defined as 1.0 µmol of SANA hydrolyzed per minute at 25°C. In another set of experiments, SF samples were pre-incubated with 1 mM *p*-aminophenylmercuric acetate (*p*-APMA, Sigma–Aldrich) for 4 h at 37°C to activate proMMPs, and the elastase activity was subsequently measured. Elastase activity of SF samples was expressed as µU/ml. To inhibit metal dependent elastase activity in the SF, SF samples were pre-incubated with 10 mM EDTA solution for 3 h at 37°C, and elastase activity was measured as described previously.

STATISTICAL TESTS

Epitope concentrations and elastase enzyme activity of KJS, OA and RA SF samples were represented by box plot graphs. The solid line represents the median, the box represents the middle 50% of values, the error bars represent the 10th and 90th percentile and individual points represent outlying values. Mean levels were reported for CII peptides, 9A4 epitope, and 5-D-4 concentrations, and elastase activity. Student's unpaired two-sample *t* test was used to test for differences in the markers measured among the patient groups. The significance level was determined *a priori* to be α=0.05. Correlations between elastase activity and both CII peptides and 9A4 epitope concentrations of SF from patients with KJS were performed by Spearman's correlation, and the coefficient of correlation was also reported.

Results

CII PEPTIDES

A total of 74% of KJS SF samples had detectable quantities of CII peptides compared to 89% in OA SF samples, and 90% in RA SF samples (Fig. 1). Postmortem normal SF samples had undetectably low CII peptide concentrations (<50 ng/ml). The CII peptides in SF from patients with KJS showed a wide range of concentrations between 0.07 and 3.37 µg/ml, a mean of 0.94 µg/ml, 95% CI (0.86–1.20 µg/ml). The OA and RA SF aspirates had means of 0.13 µg/ml, 95% CI (0.12–0.15 µg/ml) and 0.17 µg/ml, 95% CI (0.14–0.19 µg/ml), respectively. CII peptide concentrations were significantly higher in SF samples from patients with KJS compared to SF samples from either patients with OA, (P<0.001), or RA (P<0.001). In addition, CII peptide concentrations in SF from patients with RA were significantly higher than patients with OA

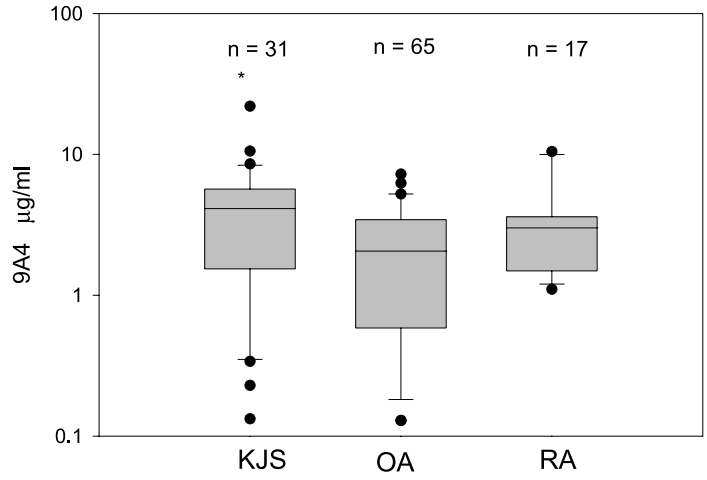

Fig. 2. Epitope 9A4 concentration by inhibition ELISA in SF from patients with KJS, OA and RA. * Epitope 9A4 concentration in KJS was significantly higher (P=0.012) than OA aspirates.

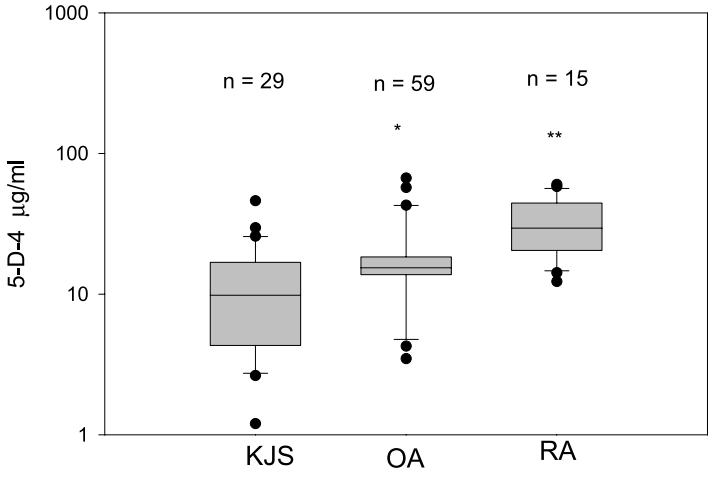

Fig. 3. Epitope 5-D-4 keratan sulfate concentration by inhibition ELISA in SF from patients with KJS, OA and RA. * 5-D-4 epitope concentration in OA was significantly higher (P<0.05) than KJS aspirates. ** 5-D-4 epitope concentration in RA was significantly higher (P<0.05) than OA aspirates.

(P<0.001). To control for variations in CII peptide concentrations resulting from differences in volume of aspirated SF samples among patient groups, hyaluronic acid and total protein were quantified (data not shown), and CII peptide concentrations were normalized to hyaluronic acid and total protein concentrations. Hyaluronic acid concentration showed no significant difference between the three patient groups. Total protein concentrations were significantly higher in patients with KJS (P<0.005) compared to OA and RA SF samples, with no significant differences between OA and RA SF concentrations. When CII peptide concentrations were normalized to hyaluronate and total protein concentrations, the significant elevation of CII peptides concentrations in patients with KJS compared to patients with OA and RA (P<0.005) was still apparent.

9A4 EPITOPE

A total of 65% of KJS SF samples had detectable quantities of 9A4 epitope compared to 78 and 85% in OA and RA SF, respectively (Fig. 2). The mean concentrations of 9A4 epitope were 4.52, 2.36, and 3.71 µg/ml for patients with KJS, OA and RA, respectively. Concentrations of 9A4 epitope in KJS SF were significantly higher than OA SF (P=0.012) but not different from RA SF concentrations (P=0.448). There were no significant differences in 9A4 epitope concentrations between SF from patients with RA and OA (P=0.119). The 9A4 inhibition ELISA is indicative of early signs of articular cartilage damage leading to the production of active collgenolytic activity, which can release collagen peptides.

5-D-4 EPITOPE

Total sGAG was measured by DMB dye-binding assay, and 5-D-4 epitope was quantified using inhibition ELISA. Mean sGAG concentrations in SF from patients with KJS were significantly lower (P<0.005) than either OA or RA samples, while there was no significant differences in sGAG SF levels between OA and RA (data not shown).

A total of 60% of KJS, 82% of OA and 75% of RA SF samples contained detectable quantities of 5-D-4 epitope

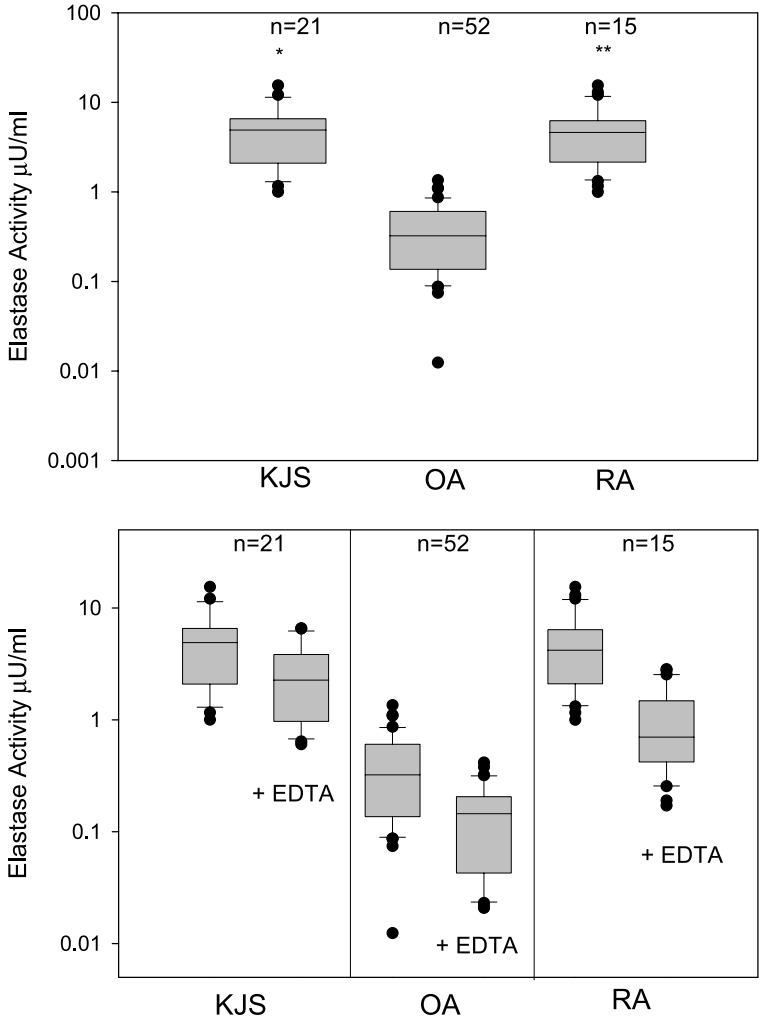

Fig. 4. Elastase activity in SF from patients with KJS, OA and RA using SANA substrate. (a) Elastase activity in SF from patients with KJS, OA and RA. * Elastase activity in KJS was significantly higher (P<0.05) than OA aspirates. ** Elastase activity in RA was significantly higher (P<0.05) than OA aspirates. (b) Inhibition of elastase activity in KJS, OA and RA SF by EDTA treatment.

(Fig. 3). SF from patients with KJS showed significantly lower concentrations of sGAG and 5-D-4 keratan sulfate epitope compared to SF from patients with either OA or RA.

ELASTASE ACTIVITY

A total of 44% of patients with KJS had detectable elastase activity compared with 72% in patients with OA and 75% in patients with RA [Fig. 4(a)]. The mean value for elastase activity in KJS aspirates was 5.12 µU/ml, compared to 0.40 µU/ml in OA aspirates and 4.99 µU/ml in RA aspirates. Elastase activity in SF from patients with KJS and RA were both significantly higher than SF from patients with OA (P<0.001) and (P<0.001), respectively. Activation of prometalloproteinases by *p*-APMA caused a significant elevation in elastase activity in SF from patients with OA (P<0.001). Although the mean values for elastase activity in KJS aspirates slightly increased, there was no significant elevation in either KJS or RA SF samples.

To further investigate the source of elastase activity in the SF samples, EDTA was added to abolish metal dependent elastase activity [Fig. 4(b)]. The mean inhibition of elastase activity by EDTA in KJS aspirates was calculated to be approximately 43% compared to 65% in OA aspirates and 80% in RA aspirates.

Correlations of elastase activity to CII peptide concentration [Fig. 5(a)] and 9A4 epitope concentration [Fig. 5(b)] were calculated to establish a relationship between elastase activity and CII degradation in SF from patients with KJS. The correlation between elastase activity and CII peptide concentration was not significant (r=0.397; P=0.128). On the other hand, there was a significant correlation between elastase activity and 9A4 epitope concentrations (r=0.583; P=0.009).

Discussion

In this study, CII peptide concentrations in the SF from patients diagnosed with KJS, OA and RA were reported using a novel sandwich, CII-specific, ELISA. The elevated CII peptide concentrations in SF from patients with KJS compared to normal controls and OA aspirates indicate early signs of cartilage network damage. Elevated 9A4

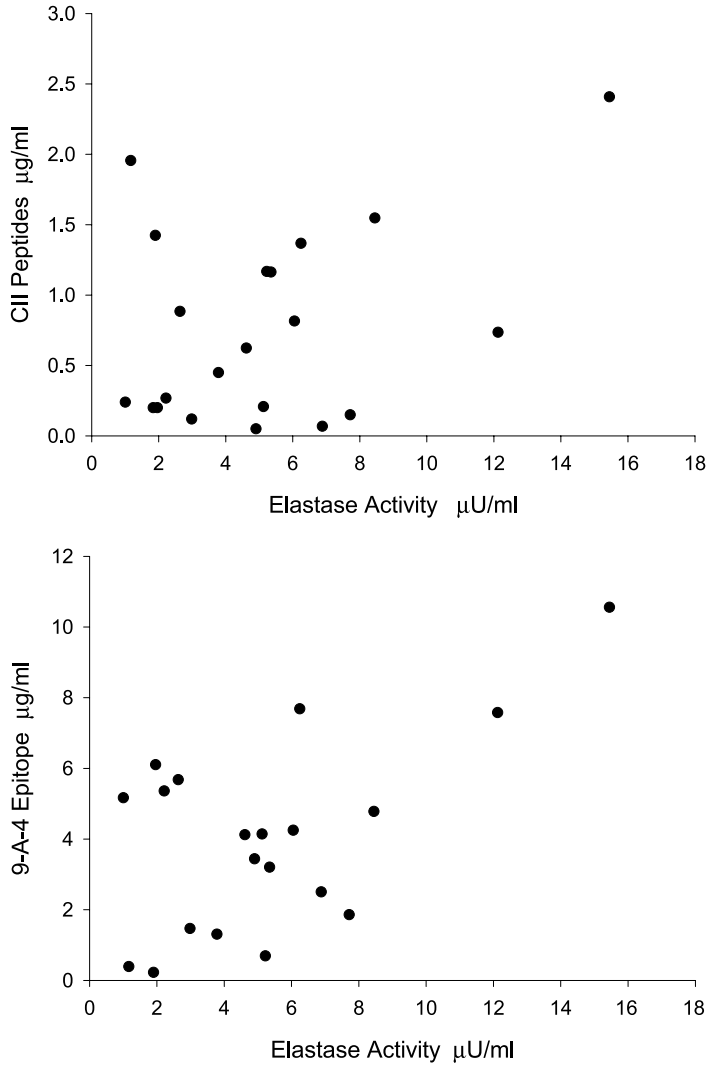

Fig. 5. Correlations between elastase activity and collagen type II epitopes concentrations in SF from patients with KJS. (a) Correlation between elastase activity and CII peptides concentrations. r=0.397, *P*=0.128. (b) Correlation between elastase activity and 9A4 epitope concentration. r=0.583, P=0.008.

epitope concentration in SF from patients with KJS indicates active metalloproteinase activity in these patients. Epitope 9A4 is produced when CI, CII, and CIII are degraded by the collagenase subfamily of MMPs[6]. The presence of elevated 9A4 epitope concentrations in SF from KJS patients suggests that MMP-1, MMP-8 or MMP-13 contribute to the degradation of cartilage matrix following injury. Elevated expression of chondrocyte MMP-13 was reported following acute injury[11], and expression of MMP-1 in traumatic synovial membrane has also been identified[15]. All these findings point to an important role of MMPs in the degradation of cartilage matrix in traumatic conditions.

The higher elastase-like activity in SF from RA and KJS compared to OA results from increased concentrations of either neutrophil elastase (NE) or MMPs in SF. NE is a serine protease that degrades connective tissue components such as elastin, and proteoglycans[16]. NE cleaves native helical CI, CIII, and CX[17]. Helical CII is resistant to cleavage by NE[17]. In addition to its direct collagenolytic activity, NE was shown to activate MMPs[18] and inactivate their tissue inhibitors[19], thus causing an imbalance that favors increased collagenolytic activity. NE also plays a significant role in the pathology and progression of RA[20].

In KJS aspirates, early elevation of metal dependent elastase activity may contribute to the CII degradation and release into the SF. The different types of collagens are arranged in a unique spatial arrangement that excludes CII from the outer layer of articular cartilage. NE, and not collagenases, was shown to be implicated in the destruction of the outer cartilage layer, exposing the underlying collagen matrix[21]. It is possibly the concerted action of NE, through destruction of the superficial layer of cartilage and degradation of proteoglycans leading to better accessibility of CII molecules, and collagenases, through specific cleavage of CI, and CII, that combined lead to damage to the superficial layer of cartilage and release of CII fragments in the SF.

The sGAG and 5-D-4 are markers of matrix turnover and degradation. The lower concentrations reported for sGAG and 5-D-4 in SF from patients with KJS suggest that injury is confined to the superficial zone of cartilage among this

population. Our sGAG measurements are in agreement with other reports that found no significant differences between sGAG concentrations in SF from patients with OA and RA[22]. Analysis of full-depth articular cartilage for its sGAG content revealed that CS content is highest in the mid-zone while KS content progressively increases through the depth of the cartilage[23]. This may explain that the low levels of release of sGAG in KJS aspirates are due to a superficial injury to the articular cartilage.

Collection of SF samples in our study was performed with extreme care. The samples were processed quickly after aspiration and stored frozen to minimize any proteolytic degradation after aspiration. Synovial effusion can lead to dilution of the cartilage degradation markers measured in this study. Therefore, hyaluronate concentration was determined, and CII peptides concentrations were normalized to hyaluronate concentration. Hyaluronate is locally produced by the synovium. Total protein concentrations were also used to identify any variations resulting from synovial effusion. The higher protein concentrations in SF from patients with KJS can be a result of infiltration of low molecular weight proteins through the synovial membrane under inflammatory conditions.

The results of this study delineate early signs of cartilage degradation in a patient group with KJS. Patients diagnosed with KJS represent a new population of study subjects to the rheumatologic and orthopedic research communities. These patients are primarily cared for by emergency physicians and do not receive routine orthopedic follow-up. These patients have not been subjected to previous cartilage degradation marker studies. They appear to have an injury that is confined to the superficial zone of articular cartilage. The cascade of events leading to the release of collagen type II peptides and aggrecan in the SF of patients with KJS is not fully understood, however, collagenases and NE play a role in the degradation process.

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