High levels of aggrecan aggregate components are present in synovial fluids from human knee joints with chronic injury or osteoarthrosis

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One new approach which has considerable potential for use in the diagnosis and prognosis of osteoarthrosis (OA) is the determination of biochemical markers in body fluids, and aggrecan, the major proteoglycan of articular cartilage, has been identified as a candidate marker (Israel et al. 1991, Lohmander et al. 1989, 1993, Ratcliffe et al. 1992, Saxne et al. 1985, 1986). In the matrix the aggrecan molecules form large aggregates by binding to hyaluronan, and this interaction is stabilized by a separate link protein. Catabolism of the aggrecan aggregates results in cleavage of the aggrecan protein core and link protein at specific sites, and the fragments are released from the cartilage into the synovial fluid. In normal tissue these are events of maintenance, but in degenerative cartilage proteoglycan catabolism occurs at accelerated rates. Previous studies have shown that the levels of specific components of the aggrecan aggregate, including the sulfated glycosaminoglycan (S-GAG), keratan sulfate (KS) and link protein, in synovial fluid can indicate increased catabolism in cartilage at early stages of OA (Israel et al. 1991, Ratcliffe et al. 1992, 1994). Recent studies have shown that the monoclonal antibody 3B3 recognizes a chondroitin sulfate epitope (termed 3B3(-)) that is expressed in OA cartilage and can be detected in synovial fluid of OA joints, but is not present in normal articular cartilage and synovial fluids (Carney et al. 1992, Caterson et al. 1990, Ratcliffe et al. 1993, Slater et al. 1992, Visco et al. 1993). The objective of this study was to use the approach of quantitative determination of specific aggrecan aggregate components in synovial fluids from patients with joint disease characterized by arthroscopy, thereby allowing an accurate comparison of the joint fluid analysis with clinical joint disease.

Methods

Human knee joint pathology characterization and synovial fluid aspiration

Synovial fluid was obtained from the knee joints of 55 patients at the time of arthroscopy or surgery for joint replacement, centrifuged to remove cells and tissue debris, and the supernatant stored at –20 °C for analysis to determine S-GAG, KS epitope, link protein and 3B3(-) epitope. Visual diagnosis of the articular cartilage by arthroscopy, and time after injury, were used to assign the patients to one of three groups: (a) acute injury, (b) chronic injury, and (c) OA. The average age of the patients in the acute injury group (n=10) was 37 (26– 61) years, the chronic injury group (n=10) was 47 (26–58) years, and the OA group (n=35) was 66 (28–80) years. Patients were considered to have an acute knee injury if the examination was within 4 weeks of the trauma and it showed no signs of OA. The injury was considered to be chronic if the examination was more than 4 weeks after the trauma, and the articular cartilage showed no signs of OA. The injuries of these patients were torn anterior cruciate ligament or torn mensicus. OA was diagnosed by direct inspection using the classification of Outerbridge (1961). All patients in this study included in the OA group were grade II, III, or IV, and all had previously been diagnosed as having OA by usual clinical symptoms and by radiography.

Articular cartilage and menisci were obtained from knee joints with OA that were undergoing surgery for joint replacement. Normal articular cartilage was also obtained from a joint of a limb undergoing amputation, and from five fresh-frozen cadaveric specimens. The tissue was extracted with 4 M guanidine HCl/0.05 M sodium acetate, pH 5.8, in the presence

of protease inhibitors, dialyzed against H_2O and analyzed to determine the level of 3B3(-) epitope. Synovial fluid samples for analysis to determine KS epitope and S-GAG levels were first digested with papain at 60 °C for 16 hours followed by heat inactivation of the enzyme at 95 °C for 30 minutes.

Quantitation of matrix components

Keratan sulfate epitope was determined by a non-competitive inhibition ELISA (Ratcliffe et al. 1994). This procedure uses Fab fragments of the monoclonal antibody 5D4, and all samples and standards are digested with papain prior to analysis. Chondroitinase ABC and papain-digested aggrecan was used as standard. The radioimmunoassay procedure used to determine concentrations of link protein was used as described previously (Ratcliffe et al. 1992). Quantitation of 3B3(-) epitope was by ELISA as previously described (Ratcliffe et al. 1993), using the monoclonal antibody 3B3 (Caterson et al. 1990), and chondroitinase ABC-digested human articular cartilage D1 was used as standard. The concentration of native epitope in the articular cartilage extracts was calculated per mg total S-GAG in the extract, and per mg hydroxyproline in the tissue.

S-GAG was determined using the 1,9-dimethylmethylene blue dye assay modified for use in microtiter plates (Ratcliffe et al. 1994). Shark chondroitin sulfate was used as the standard. To determine collagen, tissue was solubilized by digestion with papain, hydrolyzed, and assayed for hydroxyproline content using a colorimetric procedure adapted for use with a microtiter plate reader (Guilak et al. 1994).

Immunolocalization of 3B3(-) epitope in human articular cartilage and mensici

Articular cartilage and menisci from OA (n=3) and non-OA (n=3) joints were obtained immediately at surgery and was fixed in 10% neutral buffered formalin for 72 hours at 4 °C. The tissue was decalcified, dehydrated in a series of graded alcohols, embedded in paraffin, 6mm sections were cut, deparaffinized and subjected to immunolocalization with the monoclonal antibody 3B3(-) (Visco et al. 1993). Sections were pretreated with 20% sheep serum, incubated with antibody 3B3(-) (diluted 1/100), followed by incubation with goat anti-mouse Ig-biotin conjugate, then streptavidin-peroxidase, and color was developed by incubation with 0.03% H_2O_2 in 0.5 mg/ml 3,3'-diaminobenzidine tetrachloride. The sections were lightly counterstained with hematoxylin and viewed. To determine non-specific staining, no primary antibody controls were used with replicate sections. Sections presented are representative of the observations of all tissues examined by immunolocalization.

Statistical analysis

Data were analyzed through multivariate analysis of variance and a significant difference ($p < 0.05$) between the groups allowed an analysis of individual variables by univariate analysis of variance. The groups were compared using the Student-Newman-Keuls multiple range test, and statistical significance was set at $p < 0.05$. All analyses were performed using the SAS statistical software package on a VAX II/750 minicomputer.

Results

Levels of cartilage matrix components in human synovial fluids

The levels of S-GAG, KS epitope, link protein and 3B3(-) epitope in the synovial fluids of the 3 groups are shown in Figure 1. In the synovial fluids of the acute injury group, levels of S-GAG, KS epitope and link protein were all relatively low (Figure 1), and similar to concentrations of these components in synovial fluids determined in previous studies (Ratcliffe et al. 1994). No 3B3(-) epitope was detected in any of these acute injury synovial fluids. Levels of S-GAG in the chronic injury group and the OA group were significantly higher, although no difference was detected between the S-GAG levels in the fluids from the chronic injury and OA groups. The levels of KS epitope in the synovial fluids of the chronic injury group was significantly elevated, compared to the acute injury group, with some of the samples containing particularly high concentrations of epitope, and all samples having levels of KS epitope higher than the acute injury samples. The level of KS epitope in the fluids of the OA group was also significantly elevated compared to the acute injury group. These levels for these samples were in the lower range of values obtained for the chronic injury group, and the OA group value was significantly different from both the acute and chronic injury groups. The concentration of link protein in the synovial fluids of the chronic injury group was elevated compared to the acute injury group, and all of the samples had concentrations of link protein above those of the acute injury group. The concentration of link protein in the OA group was also elevated compared to the acute injury group, and no statistically significant difference could be detected between the chronic injury group and the OA group ($p > 0.1$). The 3B3(-) epitope was found to be present in all of the samples of the chronic injury and OA groups, in contrast to the acute injury group.

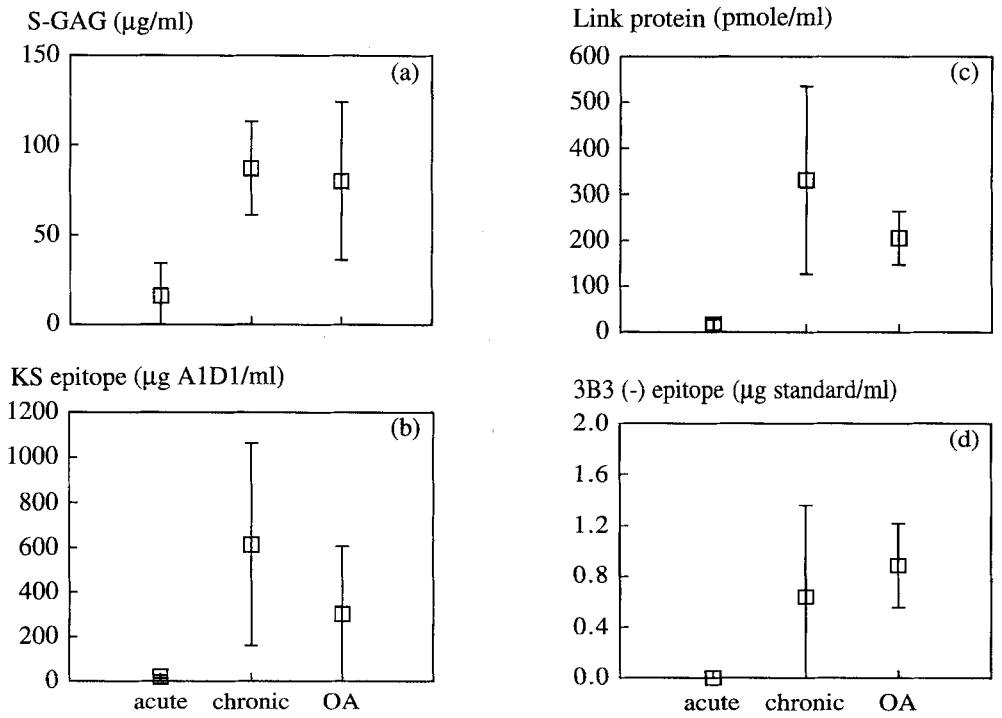

Figure 1. The concentration of (a) S-GAG, (b) KS epitope, (c) limk protein, and (d) 3B3(-) epitope in the synovial fluids of patients with joint disease diagnosed by arthroscopy. The concentration of KS epitope in the fluids from the acute injury group, the chronic injury group and the OA group were significantly different from each other. The concentration of link protein in the synovial fluids from the chronic injury group and the OA group were not significantly different from each other, but were significantly different from the acute injury group. The concentration of 3B3(-) epitope in the fluids from the chronic injury group and the OA group were significantly different from the acute injury group, but were not significantly different from each other. Data is presented as means and SD.

3B3(-) epitope in articular cartilage and menisci

Immunolocalization of 3B3(-) epitope in OA articular cartilage showed the presence of epitope throughout the depth of the cartilage (Figure 2). In cartilage with intact articular surface, the staining was pronounced, and was present throughout the depth of the cartilage (Figure 2a) but was most pronounced in the superficial zone and middle zones (Figure 2b). In cartilage with heavily fibrillated surface and/or erosion, the immunostaining in the matrix was less intense, and was present throughout the depth of the tissue (Figure 2c). In some areas the pericellular matrix surrounding the chondrocytes stained deeply (Figure 2c). Normal articular cartilage failed to show any staining (Figure 2d). Immunolocalization of the 3B3(-) epitope in menisci from joints with OA showed staining to be light, but present near the surfaces and the inner rim. Little or no staining was observed in the tissue toward the periphery.

Analysis of 4M guanidine extracts of articular cartilage from OA joints (n=6) also showed the presence of relatively high concentration of native epitope, whereas no epitope was detected in non-OA articular cartilage (Table 1). Analysis of menisci that were obtained from OA joints at the time of joint replacement showed the presence of native 3B3 epitope, but at a concentration relative to S-GAG levels that was

Table 1. The level of 3B3(-) epitope in 4 M guanidine HCl extracts of articular cartilage and menisci from knee joints with or without OA. The value of 0.05 µg A1D1/µg S-GAG or /µg hydroxyproline was considered to be the limit of detection for these analyses

Sample	3B3(-) /S-GAG µg A1D1/µg	3B3(-) /OH-Pro µg A1D1/µg	S-GAGo /OH-Pro µg/µg
Articular cartilage			
Non-OA (n=6)	<0.05	<0.05	1.56±0.68
OA (n=6)	1.15±1.16	0.93±0.55	1.14± 0.91
Meniscus			
Non-OA (n=4)	<0.05	<0.5	0.12±0.05
OA (n=6)	0.62±0.64	0.28±0.46	0.22±0.09

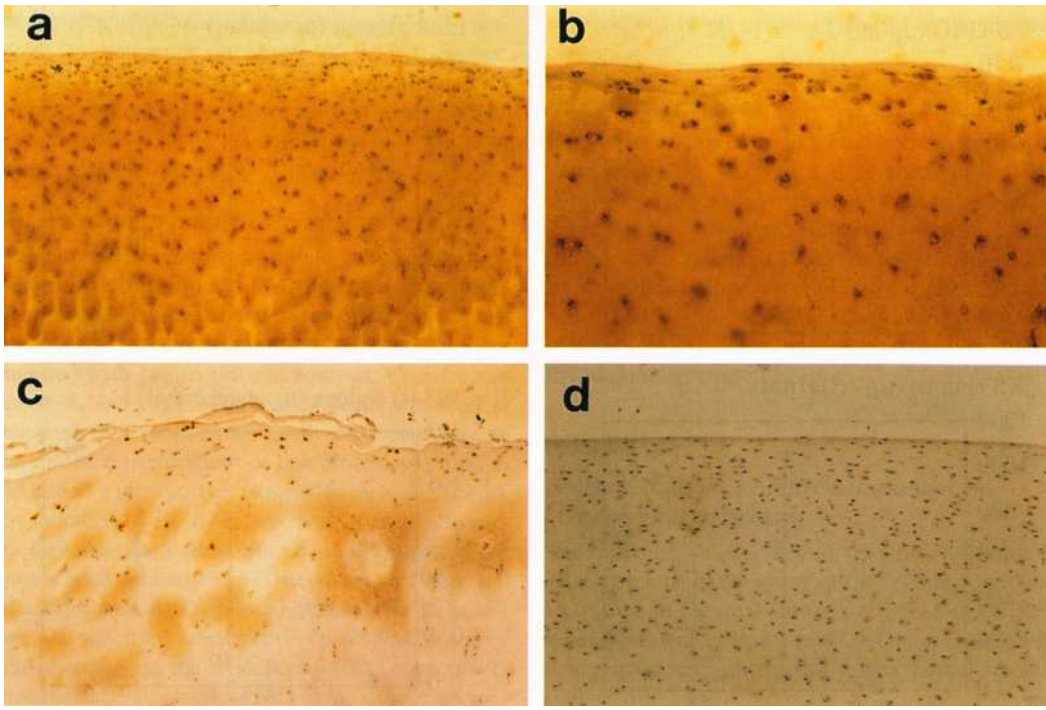

Figure 2. Immunolocalization of 3B3(-) epitope in (a) articular cartilage of non-visually involved tissue from the femoral condyle of an OA joint, showing 3B3(-) staining through the depth of the articular cartilage; (b) OA femoral condyle articular cartilage with intense 3B3(-) staining in the superficial and middle zones; (c) OA tibial plateau surface zone fibrillated articular cartilage showing reduced 3B3(-) staining in the matrix and pericellular matrix; (d) normal articular cartilage showing no staining.

1.9 fold less than that in articular cartilage, and 5.2 fold less relative to the hydroxyproline levels.

Discussion

Arthroscopic characterization of the joints in this study offers the most accurate and sensitive method of clinically assessing OA. Arthroscopic visualization was unable to distinguish between the acute injury and the chronic injury (no OA) groups, whereas biochemical analysis was able to distinguish between these groups. Increased levels of proteoglycan have been observed in synovial fluid up to 20 years after trauma (Lohmander et al. 1989, Lohmander et al. 1994), and previous clinical studies indicate that a proportion of patients with meniscal and ligament injuries are likely to progress to degenerative joint disease. In a longitudinal study (Dahlberg et al. 1994) the levels of biochemical markers in synovial fluids from joints with chronic injury were higher in those from patients with significant knee problems, as compared to patients with mild knee problems. In the present study, the samples were all obtained from patients reporting with significant knee problems, and does not take into account those with chronic injury but without clinical problems. Continued longitudinal studies are required to determine if the levels of these components in the synovial fluid could be predictive of future cartilage or meniscal degradation. The elevated levels of link protein and KS epitope, and the presence of the 3B3(-) epitope, in the fluids of the OA knees suggest that specific metabolic events are ongoing in the OA cartilage and are similar to those observed in the previous experimental studies. These results also indicate that in clinical OA, as observed in the canine experimental model of OA (Ratcliffe et al. 1992), an important event is not only the degradation of aggrecan, but also the degradation of link protein. The synovial fluids obtained from patients within 4 weeks of injury and had no arthroscopic evidence of OA showed relatively low levels of catabolic markers, which is consistent with another recent study (Dahlberg et al. 1994).

The data suggests that the 3B3(-) epitope is generated early in the disease process, and that it has excellent potential as a biochemical marker of OA. Previous experimental studies have shown that dramatic changes occur in the upper zones of articular cartilage early in the disease process (Guilak et al,

1994), including the appearance of native 3B3(-) epitope (Visco et al. 1993). Metabolic events in the upper zones of the articular cartilage are likely to be important in the early development of OA. The novel observation that 3B3(-) epitope was in the menisci of OA joints (presumably synthesized by the fibrochondrocytes of the menisci) indicates that this could make a small but significant contribution to the level of 3B3(-) epitope in the synovial fluid. The presence of 3B3(-) epitope in synovial fluid could therefore also indicate early degenerative events of the articular cartilage or the meniscus, and possibly of the other connective tissues of the joint.

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