Synovial fluid chondroitin sulphate epitopes 3B3 and 7D4, and glycosaminoglycan in human knee osteoarthritis after exercise

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Abstract

Objective—Walking exercise alleviates some symptoms, such as pain, in patients with mild to moderate knee osteoarthritis (OA). However, a major concern is that weightbearing exercise on osteoarthritic joints may exacerbate articular cartilage degradation. Loading of proteoglycan depleted articular cartilage in vitro increased expression of the chondroitin sulphate epitope 3B3, suggesting that loading may influence metabolism of osteoarthritic cartilage. This study aimed at evaluating the effects of walking exercise on articular cartilage metabolism in patients with knee OA, as reflected by changes in concentrations of synovial fluid markers.

Methods—Thirty elderly patients with knee OA (Kellgren-Lawrence grades II to IV) were randomly allocated to control (n = 15) and 12 week exercise (n = 15) groups. Synovial fluid obtained from 21 of the patients at time zero and after 12 weeks was examined by enzyme linked immunosorbent assay (ELISA) for the chondroitin sulphate epitopes 3B3 and 7D4, and by a dye binding assay with 1,9-dimethylmethylene blue for total sulphated glycosaminoglycan (GAG) concentrations. The 3B3/GAG and 7D4/GAG ratios were calculated.

Results—No significant changes in concentrations of 3B3, 7D4, GAG, 3B3/GAG, or 7D4/GAG between time zero and 12 weeks were found in either group. However, there were significant declines in 3B3 (p=0.001), GAG (p=0.007), and the 3B3/GAG ratio (p=0.049) with aging.

Conclusion—Twelve weeks of walking exercise had no demonstrable adverse effects on articular cartilage metabolism, as reflected by the concentrations of synovial fluid GAG or the chondroitin sulphate epitopes 3B3 and 7D4.

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Mild to moderate osteoarthritis (OA) of the knee has a prevalence of about 65% in the middle aged and elderly population, especially women, though progression to severe disease is uncommon.[1 2] Recommendations for management of mild knee OA include control of obesity, quadriceps strengthening exercise, and walking exercise.[1 3 4] Although walking exercise alleviates some symptoms of knee OA, including pain,[5–7] one concern is that weightbearing activity may exacerbate articular cartilage degradation.

Joint loading at physiological levels is necessary for maintenance of normal joint morphology, but overuse may be damaging. Prolonged running exercise by experimental dogs resulted in alterations in stiffness, morphology, and proteoglycan content of knee articular cartilage,[8–12] and horses engaged in strenuous exercise had reduced aggrecan and decorin synthesis in carpal articular cartilage.[13] Furthermore, female athletes engaged in weightbearing sports (competitive running and tennis) had an increased risk of developing radiological signs of hip and knee OA in later life.[14] Moreover, immediately after a single bout of exercise (running or soccer) by healthy subjects, levels of synovial fluid markers of cartilage matrix degradation were raised.[15] Although these data suggest that excessive weightbearing exercise may have damaging effects on normal joints, little is known about effects of weightbearing exercise on osteoarthritic joints.

Previously, we found that keratan sulphate concentrations in synovial fluid in osteoarthritic knees of elderly patients were unaltered by a 12 week exercise programme.[5] By contrast, keratan sulphate concentrations in synovial fluid of equine carpal joints with acute osteochondral defects and secondary OA, were raised significantly after 17 weeks of exercise.[16] This apparent disparity in findings might have been due to differences in chronicity of disease. In chronic OA, concentrations of keratan sulphate in synovial fluid are low, and unaltered by episodes of acute inflammation.[17] Some other proposed markers of altered cartilage metabolism in OA are epitopes on chondroitin sulphate chains recognised by the monoclonal antibodies 3B3 and 7D4.[18 19] Articular cartilage from osteoarthritic human knees had increased expression of these markers.[20] Also, levels of 3B3 and 7D4 in synovial fluid were raised in human knees with acute traumatic cruciate ligament and meniscal injuries.[21] Intermittent compression of bovine articular cartilage in vitro, using loading regimens within physiological range and frequency, increased the expression of 3B3.[22] This effect was more pronounced in proteoglycan-depleted cartilage, suggesting that loading may be harmful to cartilage that has pre-existing matrix damage.[23] To examine further the effects of weightbearing exercise on osteoarthritic joints in vivo, we measured 3B3 and 7D4 concentrations in synovial fluid from osteoarthritic joints of

Table 1   Demographics and characteristics of subjects with knee osteoarthritis (mean (SEM))

	Control (n=11)	Exercise (n=10)
Male:female	4:7	3:7
Age	73.1 (1.9)	66.3 (1.9)*
Body mass index	26.4 (0.7)	30.8 (1.3)†
Kellgren-Lawrence radiographic grade		
II	4	2
III	2	4
IV	5	4

*Difference between groups significant (p=0.015).
†Difference between groups significant (p=0.006).

patients undergoing a 12 week walking exercise programme. We proposed the hypothesis that increased mechanical loading of osteoarthritic articular cartilage would result in increased concentrations of 3B3 and 7D4 in the synovial fluid.

Methods

SUBJECTS

With approval of the institutional ethics committee, subjects were recruited to the study as described previously.[5] In brief, they were at least 58 years of age, and were diagnosed with stable OA, which met clinical and radiographic criteria for primary knee OA.[24] Severity of knee OA was graded radiographically by the Kellgren-Lawrence (K-L) criteria.[25] Most subjects had asymmetric bilateral disease and the knee with the highest radiographic grade was selected as the "study knee". Body mass index (BMI) was calculated according to standard criteria.[26] Subjects had no physical or medical problems for which participation in an exercise programme would have been contraindicated. They were not currently enrolled in a regular exercise programme and had not received any intra-articular or systemic glucocorticoid drug treatment within the preceding two years. Most of the subjects periodically used various analgesics for knee pain, but none routinely used any non-steroidal anti-inflammatory drugs with known potential for significantly altering cartilage metabolism.

EXERCISE PROGRAMME

After informed consent was obtained, subjects were randomly allocated to two groups: an exercise group (n=15) and a control group (n=15). The exercise programme has been previously described.[5] Briefly, the exercise group participated in a weekly health education session, and a one hour exercise session three times a week, which included quadriceps strengthening exercises and an individualised low intensity walking prescription. The control group was a delayed treatment group, and had a weekly health education class. Control subjects were instructed not to alter their routine pre-study activity levels. The duration of the study was 12 weeks.

ARTHROCENTESIS AND SYNOVIAL FLUID PREPARATION

Synovial fluid was obtained from the knee joints by arthrocentesis before and within 24 hours of completion of the intervention phase of the study. An aliquot was immediately examined microscopically, and in all samples leucocyte counts were less than 10^9/l and crystals were not seen under polarised illumination. The remaining fluid was centrifuged at 10 000 rpm and the supernatant decanted, split into aliquots, and stored at $-70°C$.

SULPHATED GLYCOSAMINOGLYCAN CONTENT OF SYNOVIAL FLUID

Aliquots of synovial fluid supernatant were digested in buffered papain solution (20 units/mg protein, Sigma Chemical Co, St Louis, MO, USA) as described previously.[21] After inactivation of the enzyme by addition of iodoacetic acid, supernatants were treated with hyaluronidase (*Streptomyces hyalurolyticus*, 2000 TRU/mg; Seikaguka Corp, Japan), and then sulphated glycosaminoglycan (GAG) levels were measured using a dye binding assay.[17 21 27] Shark chondroitin sulphate standards (Sigma Chemical Co, St Louis, MO, USA) in the range 0–40 µl/ml were prepared. After addition of 1,9-dimethylmethylene blue (Molecular Probes, Inc, USA) solution[17 27] to each plate well (Immulon 2, Dynatech Lab, Inc, USA) containing sample or standard, shifts in absorbency were detected immediately at 530 nm on a plate reader (EL 312e Bio-Kinetics Microplate Reader, Bio-Tek Instruments, VT, USA).

3B3 ASSAY

A competitive equilibrium ELISA based on a previously described method[21] was used with the following variations to quantify 3B3 epitope levels in synovial fluid. Synovial fluid samples were diluted 1:10 in saline. Standards of porcine aggrecan core protein (a generous gift from Professor Michael T Bayliss, London) in the range 1.95–500 ng/ml were prepared, and monoclonal antibody 3B3 (Seikaguka Co, Tokyo, Japan) at a dilution of 1:1600 (final dilution 1:3200) was used. Blank (Tris buffer only) and reference wells (antibody only, no competing antigen) were also included in each plate. The second antibody, antimouse IgM peroxidase conjugate (Sigma Chemical Co, St Louis, MO, USA), was diluted 1:1000. Colour reaction was read 60 minutes after addition of peroxidase substrate (ABTS, Sigma) on a plate reader at 405 nm. The 3B3 levels were calculated in equivalent weight of the antigen standard, using the absorbency compared with reference wells (no antigen), and by reading this value against the standard curve of percentage of inhibition against the standard antigen concentration.[21]

Table 2   p Values derived from analysis of synovial fluid data using split plot model for group, time, and group by time interaction, and backward elimination to determine significance of covariates (body mass index, sex, age, and Kellgren-Lawrence grade)

	Log 3B3	Log 7D4	Log GAG†	3B3/GAG	7D4/GAG
Group (control or exercise)	0.049*	0.829	0.009*	0.404	0.320
Time	0.409	0.161	0.841	0.549	0.192
Group × time interaction	0.203	0.789	0.057	0.118	0.302
Body mass index	x	x	x	x	x
Sex	x	x	x	x	x
Age	0.001*	x	0.007*	0.049*	x
Kellgren-Lawrence grade	x	x	x	x	x

*Significant effect (p<0.05).
†GAG = glycosaminoglycan.
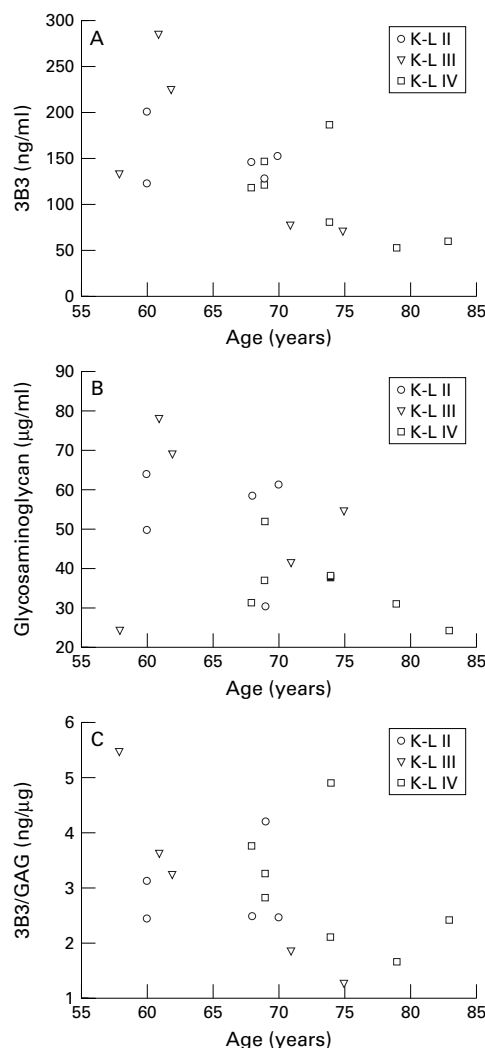x corresponds to covariates that were removed as a result of backward elimination.

Figure 1 Relation between patient age and levels of (A) 3B3, (B) glycosaminoglycan (GAG), and (C) the 3B3/GAG ratio in knee joint synovial fluid samples obtained at time zero, before the start of the study. The Kellgren-Lawrence (K-L) radiographic grade of osteoarthritis for each joint is also indicated.

7D4 ASSAY

This assay was similar to the 3B3 ELISA, with the following exceptions. Monoclonal antibody 7D4 was a generous gift of Professor Bruce Caterson (University of Cardiff). The antigen used for coating microplates and preparation of standards was bovine laryngeal cartilage proteo-glycan, generously provided by Professor Michael T Bayliss. Plates were coated at 1 µg/ml and standards ranged from 0.0156 to 4 µg/ml of sulphated GAG, as determined by the GAG dye binding assay. Synovial fluid samples were diluted 1:20 in saline. The 7D4 was applied at a dilution of 1:25 000 (final dilution 1:50 000). The second antibody IgM was diluted 1:1000. Plates were read at two hours after addition of peroxidase substrate (ABTS) on the plate reader.

DATA ANALYSIS

Age and BMI of subjects in the two groups (control and exercise) were checked to ensure normal distribution, and were compared by unpaired t tests. Data for each of the variables (3B3, 7D4, GAG, 3B3/GAG ratio, and 7D4/GAG ratio) were analysed using split plot analyses with the "mixed" procedure of SAS (SAS, Cary, NC, USA), which allowed for missing data. For each of the responses a split plot model was fitted where treatment (control or exercise) was the whole plot factor and time (zero or 12 weeks) was the subplot factor; a time by treatment interaction was also included in the model. In addition, to account for possible differences among subjects, sex, age, BMI, and K-L grade were included as covariates. Backward elimination was used to remove those covariates that did not contribute significantly to the model. Because some data were not normally distributed, all raw data were log transformed (base 10) and analyses were repeated. The "mixed" procedure was also used to construct t tests to compare group means. Values of $p < 0.05$ were considered significant. When a major result was not significant, a power calculation was performed to estimate the sample size that would be needed to show a significant difference ($p < 0.05$) 80% of the time.

Results

SUBJECTS

All 30 subjects completed the study, but synovial fluid could only be obtained from knee joints of 21 of these 30. Therefore observations and analyses for our study were restricted to this subgroup whose characteristics and demographics are summarised in table 1. There was a preponderance of female subjects in the study (67%). The control group was significantly older, and had a lower BMI than the exercise group ($p=0.015$ and $p=0.006$, respectively). The control group was in the category of overweight and the exercise group was obese.[26]

SYNOVIAL FLUID

In the backward elimination the only significant covariate was age for the variables 3B3 concentration ($p=0.001$), GAG concentration ($p=0.007$), and 3B3/GAG ratio ($p=0.049$) (table 2, fig 1). There were no significant changes in concentrations of 3B3, 7D4, GAG, 3B3/GAG, or 7D4/GAG between time zero and 12 weeks in either group (table 3). From power calculations it was estimated that for the observed differences to be significant with a power of 80% in our study, then sample sizes

Table 3 Synovial fluid 3B3, 7D4, and glycosaminoglycan (GAG) concentrations and ratios in human knee osteoarthritis after exercise (mean (SEM))†

	Time (weeks)	Control (n=11)	Exercise (n=10)
Samples analysed	0	9	8
	12	7	5
3B3 (ng/ml)	0	124.26 (24.2)	147.76 (17.8)
	12	115.87 (21.2)	111.76 (25.4)
7D4 (µg/ml)	0	62.95 (11.5)	90.24 (37.2)
	12	55.71 (8.3)	27.23 (6.5)
GAG (µg/ml)	0	47.66 (5.9)	44.35 (5.5)
	12	48.97 (7.5)*	32.84 (4.9)*
3B3/GAG ratio (ng/µg)	0	2.57 (0.3)	3.51 (0.4)
	12	2.39 (0.4)	3.44 (0.9)
7D4/GAG ratio (µg/µg)	0	1.26 (0.1)	1.76 (0.5)
	12	1.16 (0.2)	0.79 (0.1)

*At 12 weeks, difference between two groups significant ($p=0.002$).
†Split plot analyses performed on log transformed data but only the non-transformed data are shown here.

for each group of 59 (3B3), 47 (7D4), 10 (GAG), 259 (3B3/GAG), and 11 (7D4/GAG) would be needed. At 12 weeks the mean (SEM) concentration of GAG in the exercise group (32.84 (4.9) µg/ml) was significantly less than in the control group (48.97 (7.5) µg/ml) (p=0.002) (table 3). None of the other differences between groups were significant.

Discussion

Exercise is beneficial in the symptomatic treatment of knee OA, but there is concern about its effect on osteoarthritic cartilage. In our previously reported study of this group of subjects we found that visual analogue scores for pain were significantly reduced in patients undergoing a controlled exercise programme.[5] One problem with longitudinal clinical studies of OA is to quantify subtle alterations in progression of articular cartilage breakdown. Direct visualisation of articular cartilage by arthroscopy and magnetic resonance imaging is invasive or expensive. Thus there is considerable interest in attempting to define the use of synovial fluid "markers", such as 3B3 and 7D4, as indirect indicators of articular cartilage metabolism in OA. Antibody 3B3 recognises an epitope on chondroitin sulphate chains that have a non-reducing termination of GlcAβ1, 3GalNAc6S-.[28] The proportion of such terminal disaccharides, at about 9%, remains constant throughout life, but the immunoreactivity of the proteoglycan varies with the stage of development, maturity, and pathology of connective tissues, because chain length and presentation on solid phase are critical factors for recognition of chain terminations by 3B3.[28] Proteoglycans isolated from osteoarthritic cartilage and synovial fluid showed increased immunoreactivity to 3B3,[19–21] which may, in part, have been due to alterations in the sulphation and length of chains ending with the disaccharide containing the 3B3 epitope.[29] The epitope for antibody 7D4 is less well characterised, but it appears to recognise subtle combinations of sulphated and non-sulphated disaccharide isomers within the native chondroitin sulphate chain.[19] Expression of the 7D4 epitope is also more prevalent in proteoglycans extracted from osteoarthritic cartilage and synovial fluid.[19 30] It has been proposed that these are "anabolic" markers of cartilage turnover in OA, and result from attempts to repair or remodel damaged cartilage.[19] By contrast, "catabolic" markers such as BC-3, which is specific for aggrecanase cleavage in the interglobular domain, are indicative of matrix degradation.[19]

Expression of 3B3 in both normal and proteoglycan depleted articular cartilage was increased by intermittent loading in vitro, suggesting that weightbearing exercise may be harmful to joints.[22 23] An important finding of our study was that the exercise regimen had no significant effects on synovial fluid 3B3 or 7D4 epitope levels. After acute injuries of the knee, levels of 3B3 and 7D4 in synovial fluid were raised two- to threefold, and tended to be higher in the first three months after injury, and then decline.[21] This pattern may reflect an early

phase of articular cartilage matrix degradation, with clearance of these chains into the synovial fluid,[21 29] and with these markers being less sensitive indicators of chronic cartilage degradation in OA. They were not useful in discriminating between patients with chronic progressive knee OA and those with nonprogressive disease.[31]

This was the first study of temporal changes in the expression of these chondroitin sulphate synovial marker levels, evaluating the effect of exercise. Previous studies of these markers have been on single samples from each patient, and comparisons were made between patient groups.[31 32] The design of our study was statistically more powerful because patients were randomised, studied prospectively, and compared with themselves, thus eliminating the variability associated with between-patient comparisons. Although we observed a trend for the dependent variables to decline with exercise, none of these changes was statistically significant. The possibility exists that lack of significance was due to a type II statistical error, arising from small sample size or large standard deviations of data.[33] Our power calculations showed that our study was adequately powered for analyses of GAG and 7D4/GAG data, was less so for 3B3 and 7D4 data, and not at all so for the 3B3/GAG data.

One problem that evolved with our study was that the control and exercise groups differed in age and BMI at time zero baseline. Although we randomly allocated patients into the two groups at the start of the study, loss of subjects from the study due to inability to collect synovial fluid by arthrocentesis, led to this imbalance. To control for these prior group differences we analysed data using a model that included treatment, time, and treatment-time interaction, as well as subject covariates (sex, age, BMI, and K-L grade). Although we found that GAG levels in the exercise group were significantly lower than in the control group at 12 weeks, the importance of this between-group difference is unknown because of the differences in subject characteristics mentioned above. Levels of GAG in synovial fluid from osteoarthritic joints were previously reported to be lower than in normal joints.[32] The effect of exercise on synovial fluid GAG has not been studied previously, but this relation may be quite complex and influenced by other factors. For example, we found that the decline in 3B3, GAG, and 3B3/GAG ratio with age, irrespective of treatment, was also significant. This was in contrast with the observation that synovial fluid 3B3 concentration in normal knees was positively correlated with age, in a group of younger subjects.[32] These associations may relate to a change in metabolic activity of chondrocytes with aging, or perhaps advancing OA, with alterations in the sulphation and length of chondroitin sulphate chains modulating the immunoreactivity of those chains ending with the disaccharide containing the 3B3 epitope.[19–21 29 34] Although we noted a trend for older patients to have higher K-L grades (fig 1), this association was not significant. Similarly, other studies of patients with chronic

knee OA found no relation between synovial fluid markers and K-L scores, scintigraphic scan, or synovial osteocalcin levels.[32] [35] This is probably not unexpected, as bone changes do not necessarily occur in unison with cartilage degradation in OA.[31] [35]

One of the continuing controversies in synovial fluid marker studies is the influence of changes in synovial fluid volumes in joints with effusion, and the consequent dilution of any marker concentration measured. We chose to "normalise" chondroitin sulphate epitope levels against GAG, as has been done in previous studies.[31] [35] In conclusion, we found no significant deleterious effects on osteoarthritic joints, as reflected by chondroitin sulphate synovial fluid markers, in patients undergoing an exercise programme that effectively ameliorated joint pain.

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