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ORIGINAL ARTICLE

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Age-related changes and sex differences in chondroitin sulfate isomers and hyaluronic acid in normal synovial fluid

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Abstract The effects of factors such as age and sex on the metabolism of chondroitin sulfate (CS) and hyaluronic acid (HA) in knee joint tissues are believed to be profoundly important in the onset of joint diseases including osteoarthritis. To test whether age and sex influence CS isomers and HA in normal synovial fluid, we determined concentrations of chondroitin 6-sulfate (C6S), chondroitin 4-sulfate (C4S), and HA in healthy subjects of different ages. Synovial fluids were obtained from 187 healthy volunteers, 14–89 years of age. Chondroitin 6-sulfate, C4S, HA concentrations, and C6S:C4S ratio showed a significant negative correlation with age. There were no sex-related differences in HA concentration, but the concentrations of C6S and C4S and the C6S:C4S ratio were significantly lower in women than in men in most age groups.

Key words Age-related change · Chondroitin sulfate (CS) · Sex difference · Hyaluronic acid · Synovial fluid

Introduction

In recent years, determination of joint markers such as the substances originating from cartilage and synovium, various cytokines, and catabolic enzymes mainly in synovial fluid has been done in such joint diseases as osteoarthritis (OA), rheumatoid arthritis (RA), and traumatic arthritis (TA), which includes ligament and meniscus injuries. Joint markers are useful for early detection and diagnosis of the pathologic condition, stage estimation, and assessment of therapeutic effects in articular diseases.

Chondroitin sulfate (CS), the major sulfated glycosaminoglycan (GAG) in the matrix of joint tissues, plays an important role in the lubrication and nutrition of the joint.[1–3] Being a major joint marker, CS is continuously released into the synovial fluid in both normal and pathologic conditions, where its concentration and sulfation pattern reflect joint tissue metabolism.[4,5] Recently, much attention has been focused on the role of CS isomers in synovial fluid as a marker of joint disease. Chondroitin 6-sulfate (C6S) and chondroitin 4-sulfate (C4S) concentrations and the C6S:C4S ratio have been considered to change with the processes of OA and RA development.[6] In the past, we reported that CS concentration in synovial fluid reflects the stage of cartilage degeneration and the status of synovitis in OA, and is also useful as an indicator of therapeutic effects.[7–9] However, to understand the status of the disease on the basis of pathologic synovial fluid in OA and RA, accurate age- and sex-matched data of normal synovial fluid, serving as standards, are necessary. These data will be useful for understanding the status of various joint diseases, including OA. Previously, we reported age-related changes and sex-related differences in CS and HA in normal synovial fluids.[10,11] For this study, we increased our sample size and the target age groups, and conducted a more detailed determination of CS and HA concentrations in synovial fluids of healthy subjects.

Subjects and methods

Synovial fluids were obtained from the knee joints of 187 healthy volunteers with no history of knee diseases or injuries, advanced obesity, or abnormalities in lower extremity alignment. Informed consent was obtained from those who volunteered for the study in accordance with the guidelines of the research ethics committee of Nippon Medical School and Kitamurayama Public Hospital.

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Table 1. Synovial fluid concentrations of chondroitin sulfates (CS) and hyaluronic acid (HA) in healthy subjects of various ages

Age (years)	No. of subjects			Volume (ml)	C6S (nmol/ml)	C4S (nmol/ml)	C6S:C4S	HA (mg/ml)
	Total	Men	Women					
10–19	10	6	4	0.5 ± 0.3	136.7 ± 47.8	22.7 ± 7.9	6.2 ± 1.5	3.8 ± 0.7
20–29	29	19	10	0.6 ± 0.3	144.1 ± 66.2	21.0 ± 7.2	6.7 ± 1.2	3.4 ± 0.6
30–39	21	14	7	0.5 ± 0.3	120.0 ± 31.2	19.8 ± 6.3	6.2 ± 0.8	3.4 ± 0.8
40–49	15	9	6	0.5 ± 0.3	105.8 ± 30.5	18.8 ± 4.8	5.7 ± 1.1	3.2 ± 0.5
50–59	26	18	8	0.5 ± 0.3	101.1 ± 33.4	19.1 ± 5.4	5.3 ± 1.2	3.2 ± 0.6
60–69	21	11	10	0.4 ± 0.3	89.3 ± 32.3	18.4 ± 4.9	4.8 ± 1.0	2.9 ± 0.5
70–79	36	15	21	0.6 ± 0.3	72.4 ± 28.4	15.7 ± 3.3	4.6 ± 1.4	2.5 ± 0.6
80–89	29	7	22	0.6 ± 0.3	62.2 ± 26.5	14.5 ± 3.9	4.2 ± 1.1	2.1 ± 0.7
Total	187	99	88	0.5 ± 0.3	99.3 ± 47.7	18.2 ± 5.8	5.3 ± 1.4	2.9 ± 0.8

There were 99 men and 88 women with a mean age of 55.4 years (range 14–89 years). The numbers of subjects in each age group were as follows: 10 subjects in their teens, 29 in their twenties, 21 in their thirties, 15 in their forties, 26 in their fifties, 21 in their sixties, 36 in their seventies, and 29 in their eighties.

After thorough disinfection of the area around the knee joint, the synovial fluid was collected in an aseptic manner via the lateral infrapatellar approach using a 21-gauge needle. Synovial fluid samples were collected in sterile plastic tubes. After volume determination, the samples were centrifuged at 10000 × *g* for 15 min at 4°C to remove cells and joint debris. The supernatants were stored at −80°C until biochemical assay. The volume of synovial fluid required for the assay was 0.2 ml.

The unsaturated disaccharides derived from CS were measured by high-performance liquid chromatography (HPLC)[12] and those of HA by the Morgan–Elson method.[13] Chondroitinase ABC, 50 μl (5 units/ml), 10 mM sodium acetate buffer, 80 μl (pH 8.0), and distilled water, 70 μl, were added to a 200-μl volume of 10-fold-diluted synovial fluid. The mixture was incubated at 37°C for 2 h, then ultrafiltered using the Ultrafree C3GC system (molecular size cut-off 10000; Nihon Millipore, Tokyo, Japan). To completely reduce HA to the disaccharide, 30 μl of *Streptococcus dysgalactiae* hyaluronidase (0.5 units/ml; Seikagaku, Tokyo, Japan) and 30 μl of 100 mM sodium acetate buffer (pH 6.0) were added to 300 μl of the ultrafiltrate, and the mixture was incubated at 37°C for 2 h. After ultrafiltration, the mixture was analyzed by HPLC.

The unsaturated disaccharides in each sample were eluted with a gradient of 0–100 mM sodium sulfate for 60 min at a flow rate of 0.5 ml/min. The eluant from the column was mixed with 100 mM sodium tetraborate buffer (pH 9.0) containing 10 mg/ml of 2-cyanoacetamide at a flow rate of 0.5 ml/min, and the mixture was passed through a polyetheretherketone reaction coil (0.8 mm inner dimension × 10 m) set in a dry-reaction bath thermostated at 137°C. The effluent was monitored with a spectrofluorometer, set at an excitation wavelength of 331 nm and an emission wavelength of 383 nm.

We determined the reproducibility of the biochemical estimations. The coefficients of variance of the assay were 4.4% for C6S and 7.1% for C4S in ten repeated measurements of the unsaturated disaccharide standards.

Statistical analysis

The data were analyzed by Stat-View 4.02 (Brain Power, Calabasas, CA, USA), and a P value of less than 0.05 was accepted as the minimum level of significance. Multiple comparisons were performed using Scheffe's method with analysis of variance. Fisher's z-transformation of r was used for calculation of the correlation coefficient.

Results

The biochemical results are summarized in Table 1. The synovial fluid volume ranged from 0.2 to 1.6 ml (mean: 0.5 ± 0.3 ml). There was little difference in fluid volume among the age groups. The C6S concentration ranged from 23.1 to 288.2 nmol/ml (mean: 99.3 ± 47.7 nmol/ml). The C4S concentration ranged from 7.5 to 40.2 nmol/ml (mean: 18.2 ± 5.8 nmol/ml). The C6S:C4S ratio ranged from 2.2 to 9.6 (mean: 5.3 ± 1.4). The HA concentration ranged from 1.1 to 5.3 mg/ml (mean: 2.9 ± 0.8 mg/ml). The values of these markers were highest between 10 and 20 years of age, and thereafter tended to decrease.

The C6S concentration tended to decrease gradually as age increased (Table 1). A strong negative correlation with age was demonstrated, and a tendency for decreased C6S concentrations was observed in the elderly subjects (r = 0.588, $P < 0.0001$) (Fig. 1). The C4S concentration also tended to decrease with age, although the tendency was not as marked as that for the C6S concentration (Table 1). A significant negative correlation with age was shown (r = 0.409, $P < 0.0001$) (Fig. 2). The C6S:C4S ratio clearly decreased with age (Table 1). A strong negative correlation with age was observed (r = 0.591, $P < 0.0001$) (Fig. 3). The HA concentration also tended to decrease with age (Table 1). A strong negative correlation with age was observed (r = 0.615, $P < 0.0001$) (Fig. 4). We also determined sex-related differences in the C6S concentration, C4S concentration, C6S:C4S ratio, and HA concentration for each age group. In most age groups, the C6S concentration in women was significantly lower than that in men (Table 2). The C4S concentration also tended to be lower in women than in men, although the tendency was not as marked as that of the C6S concentration (Table 3). The C6S:C4S ratio was

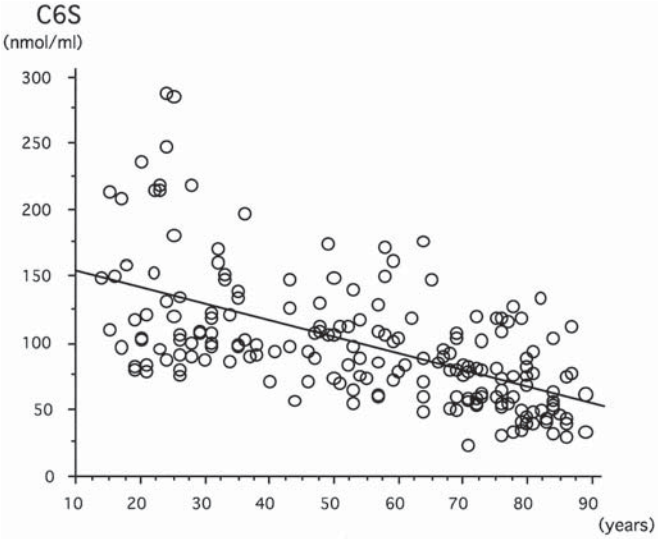

Fig. 1. The correlation of chondroitin 6-sulfate (*C6S*) concentration versus age

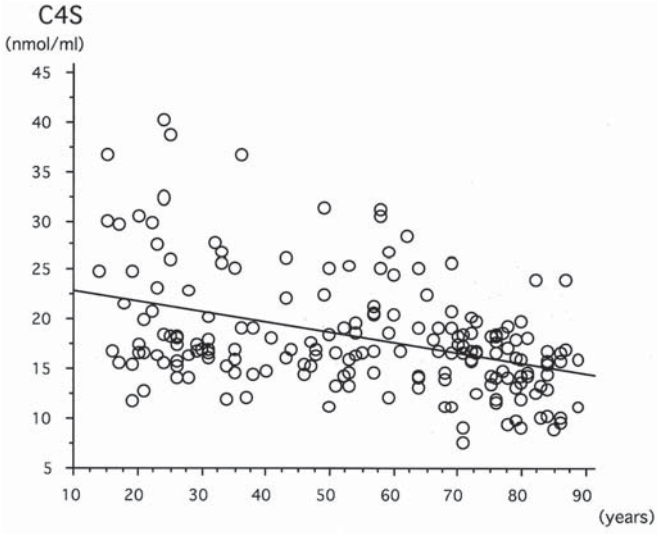

Fig. 2. The correlation of chondroitin 4-sulfate (*C4S*) concentration versus age

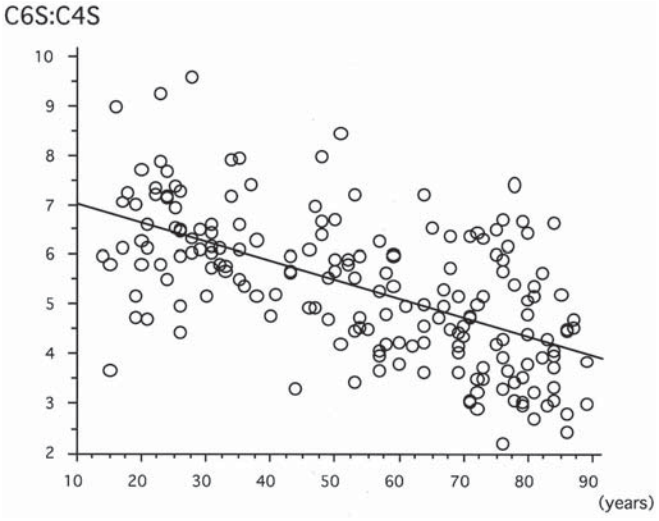

Fig. 3. The correlation of C6S : C4S ratio versus age

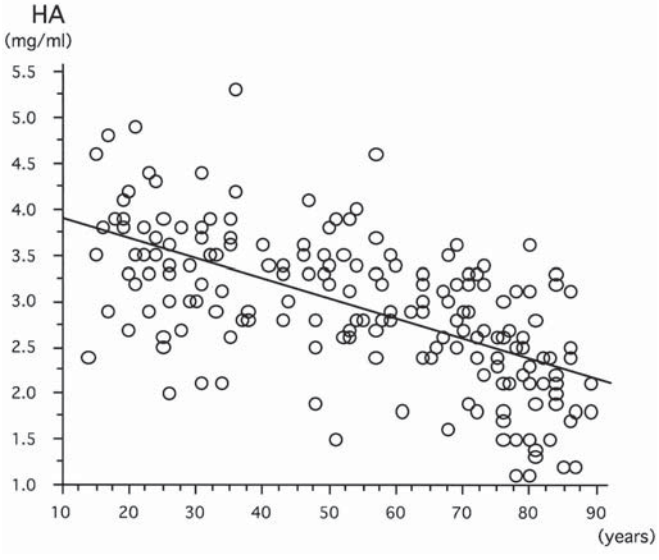

Fig. 4. The correlation of hyaluronic acid (*HA*) concentration versus age

Table 2. Differences in synovial fluid concentrations of chondroitin 6-sulfates by sex

Age (years)	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89
Men (mean ± SD)	133.1 ± 61.7 (n = 6)	162.3 ± 67.4 (n = 19)	125.2 ± 32.0 (n = 14)	119.3 ± 30.1 (n = 9)	115.3 ± 30.1 (n = 18)	93.7 ± 38.4 (n = 11)	87.7 ± 25.8 (n = 15)	76.9 ± 36.8 (n = 7)
Women (mean ± SD)	142.0 ± 21.5 (n = 4)	109.4 ± 50.2 (n = 10)	109.7 ± 28.9 (n = 7)	85.5 ± 18.5 (n = 6)	69.2 ± 10.2 (n = 8)	84.6 ± 25.1 (n = 10)	61.4 ± 25.4 (n = 21)	57.6 ± 21.3 (n = 22)
P	0.7931	0.0383	0.2946	0.0295	0.0003	0.5311	0.0045	0.0922

also significantly lower in women than in men in five out of eight age groups (Table 4). On the other hand, there were no significant sex-related differences in the HA concentration in any age group (Table 5).

These results suggest that CS concentration and sulfation pattern changes in healthy subjects are influenced by age and sex, while the HA concentration is influenced only by age. Measurements of CS isomers in synovial fluid may provide useful information for evaluating age-related changes and sex-related differences in the metabolism of cartilage.

Table 3. Differences in synovial fluid concentrations of chondroitin 4-sulfates by sex

Age (years)	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89
Men (mean ± SD)	22.3 ± 9.6 (n = 6)	22.7 ± 7.6 (n = 19)	20.9 ± 6.6 (n = 14)	19.8 ± 5.7 (n = 9)	20.5 ± 5.8 (n = 18)	17.2 ± 4.3 (n = 11)	16.2 ± 3.0 (n = 15)	16.0 ± 6.2 (n = 7)
Women (mean ± SD)	23.4 ± 5.6 (n = 4)	17.6 ± 5.4 (n = 10)	17.5 ± 5.2 (n = 7)	17.3 ± 2.8 (n = 6)	16.2 ± 2.9 (n = 8)	19.7 ± 5.4 (n = 10)	15.2 ± 3.5 (n = 21)	14.1 ± 2.8 (n = 22)
P	0.8552	0.0710	0.2514	0.3492	0.0609	0.2500	0.3662	0.2566

Table 4. Differences in synovial fluid C6S:C4S ratio by sex

Age (years)	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89
Men (mean ± SD)	6.0 ± 1.0 (n = 6)	7.0 ± 1.2 (n = 19)	6.1 ± 1.0 (n = 14)	6.1 ± 1.0 (n = 9)	5.7 ± 1.0 (n = 18)	5.4 ± 1.0 (n = 11)	5.4 ± 1.3 (n = 15)	4.7 ± 0.5 (n = 7)
Women (mean ± SD)	6.5 ± 2.2 (n = 4)	6.1 ± 1.0 (n = 10)	6.3 ± 0.5 (n = 7)	5.0 ± 1.0 (n = 6)	4.4 ± 1.0 (n = 8)	4.3 ± 0.5 (n = 10)	4.0 ± 1.2 (n = 21)	4.1 ± 1.2 (n = 22)
P	0.6467	0.0436	0.6126	0.0462	0.0037	0.0081	0.0014	0.1877

Table 5. Differences in synovial fluid concentrations of hyaluronic acid by sex

Age (years)	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89
Men (mean ± SD)	3.8 ± 0.6 (n = 6)	3.4 ± 0.7 (n = 19)	3.3 ± 0.9 (n = 14)	3.1 ± 0.6 (n = 9)	3.1 ± 0.6 (n = 18)	2.8 ± 0.6 (n = 11)	2.6 ± 0.5 (n = 15)	2.4 ± 0.7 (n = 7)
Women (mean ± SD)	3.7 ± 0.9 (n = 4)	3.6 ± 0.2 (n = 10)	3.5 ± 0.6 (n = 7)	3.3 ± 0.3 (n = 6)	3.4 ± 0.7 (n = 8)	2.9 ± 0.4 (n = 10)	2.4 ± 0.6 (n = 21)	2.1 ± 0.7 (n = 22)
P	0.7531	0.4002	0.6260	0.5716	0.1878	0.5329	0.1836	0.3012

Discussion

We previously reported changes in CS and HA concentrations in synovial fluid from the knees of OA patients with various grades of disease and in normal subjects. In this study, we increased the target age groups and the sample size to conduct more detailed examination of age-related changes and sex-related differences in CS and HA concentrations in synovial fluids from healthy subjects. Since the metabolic activity of cartilage cells in the cartilage matrix gradually decreases with age,[14,15] not only in OA patients but also in healthy subjects, the effects of aging on cartilage metabolism should be taken into consideration when examining the synovial fluid of patients. Moreover, for the purpose of making a diagnosis based on pathologic synovial fluids and evaluating therapeutic effects in joint diseases, having standard comparative values for healthy subjects at the same age as patients is important. In the past, the concentration of HA in synovial fluid was determined in various age groups, and an age-dependent decrease of HA in this concentration was reported.[16] However, there have been only a few reports on the synovial fluid CS concentration of healthy subjects with a limited sample size.[17,18] Thus, many issues remain unresolved regarding age-related changes in the CS concentration. Lewis et al.[19] determined the C6S:C4S ratio with a mean value of 5.49 in normal synovial fluids from 14 subjects between 22 and 65 years of age. This value is very close to the result from our present study, which also revealed that CS concentrations and C6S:C4S ratio were negatively correlated with age. These parameters differentiate young groups from old ones. As to the mechanism underlying age-related CS concentrations and sulfation pattern changes in normal synovial fluids, it is postulated that these changes result from differences in joint tissue turnover or the cartilage mass persisting in the joint.[14,15,20,21]

Shinmei et al.[22] suggested that C6S mainly originated from cartilage. Mourão[23] also reported that C6S accounted for more than 90% of whole CS isomers in adult articular cartilage tissues. Recently, Bayliss et al.[20,21] reported some interesting findings concerning the zonal distribution of CS isomers in normal cartilage: the C6S concentration increases from the mid-zones of the tissue toward the articular surface, and the highest concentration of C6S is found in the upper quartile of the tissue. In an animal experiment, Platt et al.[15] found that the C6S:C4S ratio for the proteoglycan newly synthesized within cartilaginous tissues decreased with age. In light of these reports, our present results appear to indicate that release of CS, C6S in particular, from cartilaginous tissues into the articular cavity is more active in younger individuals in whom the cartilage mass is large and metabolic activity on the surface of the articular cartilage is high. With advancing age, the C6S:C4S ratio for proteoglycan newly synthesized in cartilaginous tissues is also believed to decrease.[15]

The C4S concentration also tended to decrease in elderly subjects. However, the correlation with age was weaker than that with the C6S concentration. Chondroitin 4-sulfate is widely distributed not only in articular cartilage, but also

in the synovium, ligaments, and menisci.[21–23] Therefore, the C4S concentration appears to be less affected by aging than C6S, a majority of which is derived from cartilage.

Whether age-related changes in synovial fluid CS are parallel to those in articular cartilage might, in part, be determined by topographical and zonal variations of sulfation in cartilage. In addition, joint tissues as the source of synovial fluid other than articular cartilage may contribute to the CS concentration of synovial fluid.

Hyaluronic acid plays an important role in joint lubrication and the decrease of the coefficient of friction in articular cartilage, and has beneficial effects on joint tissues, including an anti-inflammatory effect and inhibition of cartilage degeneration. It also plays a positive role in cartilage repair by facilitating the growth of cartilage cells and promoting the production of the cartilage matrix. Recently, HA was reported to inhibit apoptosis of cartilage cells.[24] Balazs[16] observed that the HA concentration in normal synovial fluid decreased with age, supporting our result in this study. In an in vitro experiment examining the interactions between CS and HA, CS was reported to increase the viscosity of the HA solution.[25] According to our results, age-related decreases of CS and HA concentrations in synovial fluid will cause low viscosity of synovial fluid, thereby increasing the friction resistance of the articular cartilage. Moreover, when the concentration of HA decreases, the growth of cartilage cells and production of cartilage matrix are also inhibited, thereby reducing the lubricant capacity of cartilage. These facts provide strong evidence for the higher prevalence of OA in middle-aged and elderly people.

Sex differences have long been attributed to the higher rate of OA among women. Examining sex-related differences in our present study revealed no differences in the HA concentration while the C6S concentration, C4S concentration, and C6S:C4S ratio were significantly lower in women than in men in most age groups. These observations suggest that there are some sex-related differences in the metabolism of cartilage. Cicuttini et al.[26] determined the volume of cartilaginous tissues in the knees of healthy subjects using magnetic resonance imaging to deduce that this volume in women was significantly smaller than that in men. Moreover, Wei et al.[27] reported that cartilage from female animals had a lower concentration of proteoglycan than that from males. Difference of CS concentrations of synovial fluid between men and women will be explainable by these observations.

In summary, CS concentrations and the ratio of C6S:C4S in normal synovial fluid decreased age-dependently. A clear observation of sex difference in sulfation patterns of CS was also noteworthy. Considering that sex difference and aging possibly have an effect on the metabolism of CS and HA production in cartilaginous tissues of the knee, this study should be useful to evaluate patient status in various joint diseases, including OA.

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