Highly sensitive ELISA for determining serum keratan sulphate levels in the diagnosis of OA

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Abstract

Objective. There have been a large number of reports on alterations in the serum level of keratan sulphate (KS), a potential marker of articular cartilage degeneration in patients with arthropathy. Such studies have commonly employed ELISA using the anti-KS monoclonal antibody 1/20/5D4 (5D4-ELISA) to determine KS levels. Recently, a highly sensitive KS ELISA (HS-ELISA) kit has been developed, allowing determination of serum KS levels even in small animals, which were formerly undetectable with 5D4-ELISA. However, the effectiveness of this kit in humans has not been demonstrated. The objective of this study was to assess the usefulness of the HS-ELISA for the analysis of human serum samples.

Methods. Serum samples were collected from 28 patients with knee OA and 23 healthy volunteers. KS was determined by 5D4-ELISA and HS-ELISA, and measurements were compared with those obtained by HPLC. KS levels in serum samples with protease pretreatment were also determined by HS-ELISA.

Results. KS levels determined by HS-ELISA exhibited a better correlation with those determined by HPLC, and a higher diagnostic sensitivity for OA compared with 5D4-ELISA. Protease pretreatment of serum further improved the correlation between the values obtained by HS-ELISA and HPLC, as well as the diagnostic sensitivity of HS-ELISA for OA.

Conclusions. HS-ELISA proved useful for determining KS level in serum and the diagnosis of OA. Pretreatment of serum samples with a protease further improved the performance of HS-ELISA.

Key words: Keratan sulphate, ELISA, Serum marker, Osteoarthritis, Protease pretreatment.

Introduction

Arthropathies such as OA are disorders characterized by destruction of the articular cartilage and subsequent articular dysfunction. The principal components of articular cartilage are type II collagen and aggrecan, a large proteoglycan (PG) characteristic of cartilage. This PG plays an important role in maintaining the elasticity of articular cartilage because of its high water-holding capacity. When articular cartilage is injured due to inflammation and increased load, aggrecan is decomposed by aggrecanases and various matrix metalloproteinases [1–3]. Although X-ray examination is the standard means of diagnosis of OA [4], it cannot detect early-stage alterations or slight injury of the articular cartilage. MRI is effective in detecting injury to cartilage, but is not useful for screening because of its high cost. A more sensitive and non-invasive means of diagnosis of OA is thus required.

Many substances have been investigated as biomarkers of cartilage destruction [5–8]. Several studies have focused on PGs or PG components released from cartilage, since degradation or destruction of cartilage is associated with the disappearance of PGs [8, 9]. The core protein of aggrecan in articular cartilage has many chondroitin sulphate and keratan sulphate (KS) chains. Many authors have reported KS as a potential marker of

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Submitted 22 June 2009; revised version accepted 29 September 2009.

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cartilage destruction [10–13]. Although some of them found that serum levels of KS were increased in animal models, reflecting cartilage destruction [10, 11], others reported that serum KS levels in humans varied within a wide range, and exhibited little correlation with the clinical or radiological features of patients with OA of the knee [13]. These studies employed ELISA using the anti-KS monoclonal antibody 1/20/5D4 (5D4 antibody) to measure KS. The basic structure of this GAG is a repeat of two carbohydrates, N-acetylgalactosamine-6-sulphate and galactose or sulphated galactose. It is known that the sensitivity of ELISA using 5D4 antibody (5D4-ELISA) depends on the degree of sulphation and the length of KS chains, because of the antigen specificity of the antibody [14].

In this regard, Yamada et al. [15] developed an assay that is affected by neither the degree of sulphation nor the length of KS chains. In this method, KS in SF is digested into two disaccharide isomers by keratanase II (KSase II), and these two disaccharide isomers are determined by HPLC. Yamada et al. found that the pattern of sulphation of KS in SF varied with the progression of OA in patients with OA of the hip. Kurahashi et al. [16] measured KS in serum using a method like that of Yamada et al., in which serum is digested by a protease before degradation of KS by KSase II, and KS is quantified by HPLC. They reported that the pattern of sulphation of serum KS chains in humans exhibits wide inter-individual variation. Kurahashi et al. recommended the use of HPLC, which allows accurate quantification of KS, rather than ELISA, which can be affected by the degree of sulphation of KS chains. We [17], therefore, used the method developed by Kurahashi et al. to measure serum KS in patients with OA of the knee or knee trauma arthropathy, and found that serum level of KS was particularly increased in the early stages of articular cartilage degradation, and suggested that serum level of KS may be a suitable test for screening of subjects suspected to have articular cartilage degeneration. However, for measuring numerous samples, an easier and accurate method of determination that requires less time than HPLC is required.

Although 5D4-ELISA has traditionally been used to measure KS in human serum and SF, it features low sensitivity for KS, particularly low-sulphate KS. Recently, a highly sensitive KS ELISA (HS-ELISA) kit has become commercially available, and enables measurement of KS, and particularly low-sulphate KS, with greater sensitivity. It has been proven to be sensitive in the measurement of KS in small animals, but not in humans. This study was performed to determine whether this HS-ELISA is useful for measuring KS in human serum.

Determination of KS by ELISA has been performed using diluted serum samples. KS in serum is bound to the PG core protein as a constituent of PG structure, whereas the standard KS for ELISA is free KS purified from cartilage. We therefore used diluted serum samples and protease-pretreated serum samples to examine the effects of structural differences of KS on the results of HS-ELISA.

Patients and methods

Samples

This study was approved by the institutional ethics committee of Marunouchi Hospital and conducted in accordance with the Declaration of Helsinki in 1975, as revised in 1983. Twenty-three volunteers [mean age 35 (range 23–52) years] and 28 patients with OA of the knee [mean age 55 (range 40–80) years] participated in the study. After obtaining written informed consent, serum samples were collected and stored at −80°C until analysis. The volunteers were healthy with no gross obesity, inferior limb malalignment, history of knee injury or knee disorders. For OA patients, X-ray images were assessed using the Kellgren and Lawrence (KL) grading scale [18]. Thirteen OA patients with KL Grade 0 or I underwent arthroscopic debridement. Fifteen OA patients with KL Grade II, III or IV underwent arthroscopic debridement, a high tibial osteotomy or total knee replacement.

Materials

In the HPLC analysis, actinase E (Kaken Pharmaceutical, Tokyo, Japan), Q Sepharose (Amersham Pharmacia Biotech Corporation, Uppsala, Sweden) and keratanase II (Seikagaku Biobusiness Corporation, Tokyo, Japan) were used to treat serum samples before the assay. In addition, β-galactosyl-(1-4)-6-O-sulpho-N-acetylgalactosamine (M-KS) and β-6-O-sulphogalactosyl-(1-4)-6-O-sulpho-N-acetylgalcosamine (D-KS), used as the standards in HPLC, were prepared by Seikagaku Corporation. The anti-KS monoclonal antibody (1/20/5-D-4), highly sensitive KS ELISA kit and KS derived from bovine cornea (KSBC) were purchased from Seikagaku Biobusiness Corporation. KS derived from shark cartilage (KPS) was supplied by Seikagaku Corporation. The D-KS/ (M-KS + D-KS) values of KSBC and KPS were 0.42 and 0.96, respectively.

HPLC analysis

HPLC was performed as previously reported [16, 17]. Each serum sample was digested with actinase E. The KS-containing fraction was extracted by Q Sepharose column chromatography and then it was desalted on a Sephadex G-25 column. Thereafter, it was concentrated, and a 0.15-ml aliquot of the sample was incubated with 0.04 ml of 100 mmol/l acetic acid buffer (pH 6.0) and 0.01 ml of 100 mU/ml KSase II at 37°C for 3 h. The reaction mixture was ultrafiltered using Nanosep (molecular weight cut-off 10 K; Pall, NY, USA) to prepare the sample for HPLC analysis. M-KS and D-KS separated by HPLC were reacted with 1% 2-cyanoacetamide/100 mmol/l sodium tetraborate buffer (pH 9.0) at 145°C, and detected using a fluorescence detector (excitation: 331 nm; emission: 383 nm). A pump (Model PU-2080; Jasco, Tokyo, Japan), a column packed with YMC gel PA-120 (7.6 i.d. x 150 mm; YMC, Kyoto, Japan), a gradient unit (Model LG-2080-02; Jasco), an autosampler
(Model AS-2059; Jasco), a fluorescence detector (Model FP-2025; Jasco), a heat reaction bath (DB-5; Shimamura Instrument, Tokyo, Japan) and analysis software (Borwin-HSS2000; Borwin, Ijsselstein, The Netherlands) were used for HPLC analysis. The flow rate was set at 0.5 ml/min and sodium sulphate was linearly increased from 0 to 100 mmol/l over 45 min. The sum of M-KS and D-KS was considered as the total amount of KS.

ELISA method

The sandwich-5D4-ELISA method proposed by Kongtawelert et al. [19] was used. A 0.05-ml aliquot of serum diluted 50-fold with phosphate-buffered saline (PBS) was added to each well coated with 5D4 antibody. Standard solutions of 0–40 ng/ml KPS prepared with PBS were also added to the wells. After incubation at 37°C for 1 h, the reaction solution was removed from the wells, which were washed with 0.05% Tween/PBS. Then, 0.025 ml each of horse radish peroxidase (HRP)-conjugated streptavidin solution and biotinylated antibody solution were pipetted into each well, mixed, and incubated at 37°C for 1 h. The reaction solution was removed from the wells, which were washed with 0.05% Tween/PBS. A 0.05-ml aliquot of substrate solution was pipetted into each well, and the plates were allowed to stand at room temperature for 10 min, followed by the addition of 1 mol/l hydrochloric acid solution to stop the reaction. The absorbance at 450 (reference wavelength 630–650) nm was determined.

HS-ELISA was performed according to the instructions provided by the manufacturer. Briefly, a 0.1-ml aliquot of the buffer supplied with the kit was added to each well pre-coated with anti-KS antibody. A 0.02-ml aliquot of serum or actinase E-pretreated serum, which was diluted 1000- and 400-fold, respectively, with buffer, was added to each well. The standard solution supplied with the kit was diluted to concentrations of 0–4 ng/ml with buffer, and then added to the wells. After incubation at 37°C for 1 h, the reaction solution was removed from the wells, which were washed with the washing solution supplied with the kit. A 0.1-ml aliquot of HRP-labelled antibody solution was added to each well, mixed, and incubated at 37°C for 1 h. Then, the reaction solution was removed from the wells, which were washed again with the washing solution. A 0.1-ml aliquot of the substrate solution was added to each well, and the plates were allowed to stand at room temperature for 30 min, followed by addition of the reaction stop solution supplied with the kit. The absorbance at 450 (reference wavelength 630–650) nm was determined.

Reaction curves with KPS and KSBC

Reaction curves with KPS and KSBC were assessed by 5D4-ELISA, whereas those with the standard KS supplied with the kit, KPS and KSBC were assessed by HS-ELISA.

Measurement of KS in protease-pretreated serum samples by ELISA

A 20-µl aliquot of protease-pretreated serum was incubated with 80 µl of water and 10 µl of 2.0% actinase E at 55°C overnight. The reaction mixture was then diluted 400-fold with the buffer supplied with the kit and heated at 100°C for 10 min. Thereafter, it was cooled in an ice bath, and KS was measured by HS-ELISA.

Statistical analysis

The Wilcoxon rank-sum test was used for the analysis of differences in serum KS between healthy volunteers and OA patients, with P-value < 0.05 considered as statistically significant. The correlations between serum KS measured by HPLC and those measured by various ELISA methods were determined by Pearson’s correlation test.

Results

Reaction curves prepared with KPS and KSBC

The levels of KSBC and KPS corresponding to an absorbance of 1.0 were 922 and 25.5 ng/ml with 5D4-ELISA (Fig. 1A) and 9.1 and 2.1 ng/ml with HS-ELISA (Fig. 1B).

Fig. 1 Reaction curves obtained with KS standards with different degrees of sulphation. Reactivities with KSBC, KPS and the standard KS supplied with the HS-ELISA kit were compared. (A) Reactivities with KSBC and KPS determined by 5D4-ELISA. (B) Reactivities with KSBC, KPS and the standard KS supplied with the HS-ELISA kit determined by HS-ELISA. Reaction curves with KSBC and KPS determined by HS-ELISA were more sensitive than 5D4-ELISA, and reactivity with KSBC was markedly improved.
respectively. Compared with 5D4-ELISA, HS-ELISA exhibited ~100-fold higher reactivity with KSBC and ~10-fold higher reactivity with KPS. The ratio of the level corresponding to an absorbance of 1.0 of KSBC to that of KPS was about 36 with 5D4-ELISA and 4.3 with HS-ELISA.

With HS-ELISA, the reaction curve with the standard KS supplied with the kit was similar to that with KPS. The standard KS supplied with the kit was therefore selected as the standard for measurement of serum KS by HS-ELISA.

**Fig. 2** Correlations between serum KS levels determined by HPLC and those by ELISA. KS levels in serum samples from 23 healthy volunteers and 28 OA patients were measured by HPLC and two ELISA methods and the correlations between results obtained using these methods were determined. (A) Correlation between HPLC and 5D4-ELISA using serum samples. (B) Correlation between HPLC and HS-ELISA using serum samples. (C) Correlation between HPLC and HS-ELISA using protease-pretreated serum samples.

**Correlation between results with ELISA methods and HPLC**

Figure 2 shows the correlations of KS values between HPLC and each ELISA method obtained in serum samples from healthy volunteers and OA patients. There was a weak correlation of measurements between 5D4-ELISA and HPLC (Fig. 2A), whereas a good correlation was found between HS-ELISA and HPLC (Fig. 2B). Pretreatment of serum samples with protease improved

**Fig. 3** Keratan sulphate levels measured by HPLC and two ELISA methods in healthy volunteers and OA patients. Individual serum KS levels and the mean (± S.D.) are shown. Individual serum KS levels in OA patients were divided to the early OA (E; KL Grade 0 or I) and the advanced OA (A; KL Grade II, III or IV). (A) Serum KS levels determined by HPLC in healthy volunteers and OA patients. (B) Serum KS levels determined by 5D4-ELISA in serum from healthy volunteers and OA patients. (C) Serum KS levels determined by HS-ELISA in serum from healthy volunteers and OA patients. (D) Serum KS levels determined by HS-ELISA in protease-pretreated serum from healthy volunteers and OA patients.
the correlation of measurements between HS-ELISA and HPLC compared with serum without protease pretreatment (Fig. 2C).

Comparison of serum KS levels measured by HPLC and ELISA between healthy volunteers and OA patients

Serum KS levels in healthy volunteers and OA patients, determined by HPLC and different ELISA methods, are shown in Fig. 3 and Table 1. The mean serum KS level in OA patients determined by HPLC was significantly higher than that in healthy volunteers. No significant difference was observed between the means of serum KS levels determined by 5D4-ELISA. The mean serum KS level in OA patients was significantly higher than that in healthy volunteers when determined by HS-ELISA with or without protease pretreatment.

When serum KS levels that exceeded the mean (2 s.d.) of the value in healthy volunteers were defined as OA positive, the diagnostic sensitivity of HPLC for OA was highest, followed by HS-ELISA of protease-pretreated serum, with no significant differences among methods.

Discussion

Serum KS levels were measured by two types of ELISA and compared with those obtained by HPLC, which is affected by neither the degree of sulphation nor the length of KS chains. Measurements by 5D4-ELISA correlated weakly with those obtained by HPLC, and exhibited no significant difference between OA patients and healthy volunteers. On the other hand, HS-ELISA measurements exhibited a good correlation with those obtained by HPLC, and the finding of a significant difference in serum KS levels between OA patients and healthy volunteers suggested greater usefulness than that of 5D4-ELISA.

Mehmet et al. [14] reported that the 5D4 antibody used in 5D4-ELISA was highly specific for KS chains of hexa- or larger oligosaccharides and/or polysulphated KS but less reactive with low-sulphated KS. We compared the reactivity of the assay with KSBC (a low-sulphated KS) and KPS (a high-sulphated KS) to characterize the antibody used for HS-ELISA. The reactivity of HS-ELISA with either KS chain was higher than that of 5D4-ELISA, indicating that HS-ELISA is useful for quantitating low levels of KS in serum. The ratio of KSBC to KPS levels at the absorbance of 1.0 was 4.3 with HS-ELISA, in contrast to about 36 with 5D4-ELISA. This result showed that HS-ELISA exhibited higher reactivity even with low-sulphated KS chains. Kurahashi et al. [16] determined the degree of sulphation of serum KS in 10 healthy subjects by HPLC, and reported that M-KS was predominant in KS chains in serum, since the mean M-KS/D-KS ratio was 3.8, and that the degree of sulphation of KS chains was relatively low. The higher reactivity of HS-ELISA with low-sulphated KS chains compared with 5D4-ELISA may explain the better correlation between HS-ELISA and HPLC measurements.

Use of protease-pretreated serum samples improved correlation with measurements obtained by HPLC, and the sensitivity of HS-ELISA for OA was comparable with that of HPLC. In serum, most of the KS chains reacting with KS antibodies are bound to a protein moiety of aggrecan or to those of decomposed aggrecan fragments. On the other hand, the standard KS supplied with the ELISA kit consists of free KS chains, the structure of which therefore differs from that of KS chains in serum samples. To overcome this difference in the structure of KS chains, serum samples were pretreated with a protease. As a result, the KS levels in protease-pretreated serum measured by HS-ELISA were ~6- to 4-fold lower than those measured in serum without protease pretreatment, and those measured by 5D4-ELISA were all below the limit of measurement (<78 ng/ml; data not shown).

Possible reasons for this decrease in measured KS levels include the following. The D-KS/(M-KS + D-KS) ratio in KSBC was 0.42, whereas that in serum KS chains was 0.21 when calculated using the mean M-KS/D-KS ratio of 3.8 obtained in 10 normal human serum samples by Kurahashi et al. [16]. These values indicate that the degree of sulphation of KS chains in human serum was lower than that of KSBC. On the other hand, high-sulphated KPS or its equivalent was used as the standard KS in each ELISA. Therefore, reactivity with standard KS is stronger than KS chains in serum or KSBC, and may result in serum KS levels lower than those measured by HPLC, which are not influenced by

### Table 1: KS levels measured by HPLC and two ELISA methods in serum from healthy volunteers and OA patients

<table>
<thead>
<tr>
<th>Assay method</th>
<th>Sample</th>
<th>Serum KS level, ng/ml</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Healthy volunteers</td>
<td>OA patients</td>
<td>P-value</td>
<td>Diagnostic sensitivity for OA, %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n = 23)</td>
<td>(n = 20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>Serum</td>
<td>913 (148)</td>
<td>1381 (238)</td>
<td>&lt;0.001</td>
<td>68</td>
</tr>
<tr>
<td>5D4-ELISA</td>
<td>Serum</td>
<td>190 (64)</td>
<td>242 (63)</td>
<td>NS</td>
<td>7</td>
</tr>
<tr>
<td>HS-ELISA</td>
<td>Serum</td>
<td>861 (245)</td>
<td>1321 (338)</td>
<td>&lt;0.001</td>
<td>39</td>
</tr>
<tr>
<td>HS-ELISA</td>
<td>Protease-pretreated serum</td>
<td>159 (62)</td>
<td>344 (109)</td>
<td>&lt;0.001</td>
<td>64</td>
</tr>
</tbody>
</table>

Serum KS levels are presented as the mean (s.d.). Diagnostic sensitivity is expressed as the percentage of OA patients whose serum KS level exceeded the mean (2 s.d.) in healthy volunteers. NS: not significant.
the degree of sulphation. The serum levels determined by HS-ELISA would be 4.3-fold lower even if the sulphation of KS chains in serum were equal to that of KSBC, and would be even lower if their degree of sulphation were lower than that of KSBC. However, the measurements in serum without protease pretreatment were comparable with those obtained by HPLC. Some of the large number of KS chains bound to the core protein of aggrecan react with the solid-phase antibody in serum samples without protease pretreatment. Therefore, KS chains that do not directly react with the antibody could be trapped in the solid-phase antibody through binding to the protein, resulting in apparently higher KS levels.

Determination of serum KS levels by HS-ELISA is a simple and useful method for the diagnosis of arthropathy, but requires validated in future studies using large, statistically representative numbers of samples. The sugar chains other than KS that compose PG may also be examined as potential biomarkers of various diseases. Pretreatment of samples with a protease, as performed in this study, may be useful for the measurement of such sugar chains.

Conclusions

HS-ELISA proved useful for the determination of KS in human serum. We also showed that KS levels measured by HS-ELISA in serum pretreated with a protease exhibited a good correlation with those measured by HPLC, and that the sensitivity of such levels in the diagnosis of OA were comparable.

Rheumatology key messages

- Serum KS measurements by HS-ELISA correlated with those by HPLC.
- HS-ELISA exhibited high sensitivity for OA.
- Protease pretreatment of serum increased performance further.

Acknowledgement

We thank Seikagaku Corporation for providing M-KS, D-KS and KPS.

Disclosure statement: S.W. has received a grant for another study from Seikagaku Corporation. All other authors have declared no conflicts of interest.

References