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ORIGINAL ARTICLE

Measuring Sulfated Glycosaminoglycans in Gingival Crevicular Fluid Based on Dimethylmethylene Blue

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SYNOPSIS

The information about periodontal conditions is necessary for regeneration of periodontal tissues. The measurement of sulfated glycosaminoglycans (S-GAGs) in gingival crevicular fluid (GCF) is important in the search for disease biomarkers. To analyze the amount of S-GAGs in GCF quickly and accurately, we have modified a microplate method previously used for urinary S-GAGs to estimate the content of S-GAGs in GCF without interference of other GCF components. GCF samples were collected from six maxillary anterior teeth and diluted GCF samples were divided into two groups (20 µl each), with or without chondroitinase ABC treatment, mixed with a dimethylmethylene blue (DMB) dye solution (180 µl) to detect S-GAG. Absorbance at 540 nm was measured using a microplate reader and the amount of S-GAG was calculated from calibration curves for standard S-GAGs where a linear relationship existed in the concentration range (0-3 µg/100 µl). Other GCF components such as glycoprotein and serum constituents did not influence the measurement of S-GAGs. Although trace amounts of S-GAGs were found in controls with a healthy periodontium (0.05 µg/100 µl ± 0.04, mean±S.E., n=5), considerable amounts of S-GAGs were found in patients with advanced periodontal disease (0.42 ± 0.11, n=6, $p<0.05$). The accuracy of this simple and rapid method of measuring S-GAGs in GCF makes it useful for screening periodontal conditions.

Key words: *chondroitin sulfate, glycosaminoglycans, gingival crevicular fluid, dimethylmethylene blue*

INTRODUCTION

Periodontal diseases are obstacles to the regeneration of periodontal tissues. The disease biomarkers are necessary for clinical diagnosis of periodontal diseases.

Glycosaminoglycans (GAGs) are the polysaccharides composed of uronic acid with hexosamine, and they are combined with various proteins in various organs and connective tissue. Significant amounts of sulfated glycosaminoglycan (S-GAG) especially chondroitin sulfate with non-sulfated hyaluronan, have been identified in the gingival crevicular fluid (GCF) of sites with severely inflamed periodontal tissues but there are few S-GAG in the healthy periodontium [1, 2, 3]. Although the GAG measuring method is used frequently, there are few reports on convenient and safe methods for rapid measurement, like the screening inspection of GAG in GCF, except for the report of Yamamoto et al.[4] using alcian blue staining. We decided to use the DMB method[5], which is better suited for urinary screening for mucopolysaccharidoses than the alcian blue assay[6] in this study. Moriyama[7] improved the method in order to adapt the small volume of urinary sample using a microplate reader. Furthermore, we added some improvements to the method so as to not be influenced by other substances, in particular proteins, and to be suitable for quick and accurate analysis of S-GAG in GCF in a manner that will be useful for the clinical diagnosis of periodontal disease.

MATERIALS AND METHODS

GCF was collected from a group of six patients aged between 20 and 63 (mean 47) years old. The patients were clinically diagnosed as having advanced periodontitis, with a Gingival Index at the test sites of greater than 2 and a Periodontal Index of greater than 4, and they were undergoing initial periodontal treatment at Osaka Dental University Hospital. GCF was also collected from a control group of five dental students with healthy periodontal tissues, who were aged between 23 and 25 years. The collection sites for both groups were the labial and palatal gingival margins of the six maxillary anterior teeth.

Two hours after brushing, the sites were cleansed with water and air-dried. GCF was collected into 2 µl capillary tubes (Microcaps, Drummond Scientific Co., Panama) for 20 min, during which time 2 µl of saline solution was applied to the collection area at approx. 5 min intervals. The fluids collected from each individual were pooled and stored at -20°C until analysed. The procedure was repeated three times for each participant on different days. The total volume of the GCF sample was diluted to 30 µl with physiologic saline in analysis. Each of 15 µl samples was pipetted onto a microplate.

We used a simple microassay system with 1, 9, dimethylmethylene blue (DMB, Merk, Germany) assay[7] that employs colorimetric determinations and a Microplate reader (Bio-Rad, New York, USA) to detect S-GAG. We used the method described by de Jong et al.[6], in that the sample was added to DMB-Tris agent and stirred, and then the absorbance was analysed. This was done to remove any component interference from the detection of S-GAG. Reagents related to the DMB assay in this study are as follows: the preservative DMB reagent is DMB 55 mM / L formic acid buffer (pH 3.3) and Tris solution is Tris (base) 2M / L. Both solutions were mixed with the DMB-Tris agent as DMB solution 10 : Tris solution 1 (pH 8.8).

Each of 15 µl samples on a microplate received 5 µl of the enzyme solution, Tris-HCl buffer, pH 8.0, with or without chondroitinase ABC (Seikagaku Kogyo, Tokyo, Japan) (Table 1). The standard samples of sulfated GAG, chondroitin-4-sulfate, chondroitin-6-sulfate, and dermatan sulfate (Seikagaku Kogyo), were also prepared to make references using the above method. A total of 20 µl sample was added to 180 µl DMB-Tris reagent and shaken to read the absorbance at 540 nm immediately using the

Table 1 Procedure of S-GAG obtained from GCF by DMB microassay system used digestion with S-GAG-specific enzyme

	GCF samples (collected with physiological saline solution)		Standard samples (dissolved in physiological saline solution)	
	contrast	digestion	contrast	digestion
Sample (μl)	15	15	15	15
Enzyme solution (μl)	-	5	-	5
Control solution (μl)	5	-	5	-
	allowed to stand for 30 minutes			
DMB-Tris reagent (μl)	180	180	180	180
Absorbance (ABS_{540})	ABS_{GO}	ABS_{GE}	ABS_{SO}	ABS_{SE}

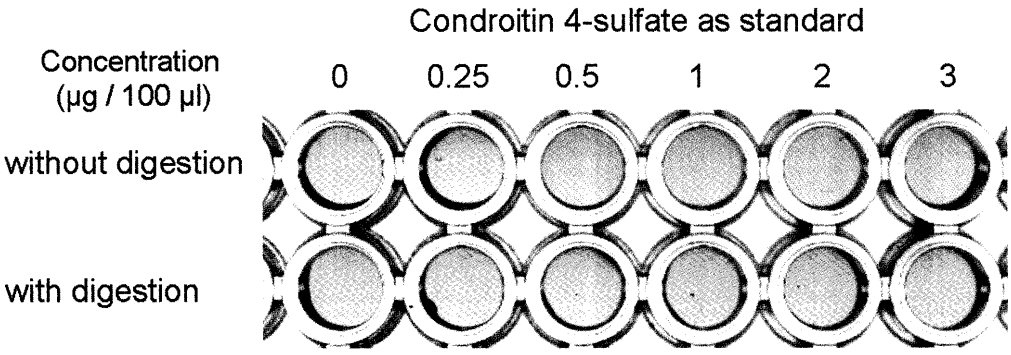
Enzyme solution : 5 units chondroitinase ABC / 500μl control solution

Control solution : 1 mg BSA / 500 μl Tris - HCl buffer (pH 8.0)

Standard sample : 0-3 ng chondroitin 4- or 6-sulfates or dermatan sulfate / 100 μl saline

(ABS_{SO} - ABS_{SE}) → made working curves

(ABS_{GO} - ABS_{GE}) →determined the amount of S-GAG in the sample



Enzyme was chondroitinase ABC (Seikagaku Kogyo, Tokyo, Japan) with Tris-HCl buffer pH 8.0

Fig. 1 Color reaction of GAGs with DMB

Microplate reader. The obtained absorbance values from standard samples with the digestion of chondroitinase ABC were taken from the values of standard samples without digestion. The obtained data were then used for standard working curves. The obtained absorbance values from GCF samples with the digestion of chondroitinase ABC were taken from the values of GCF samples without digestion. The obtained data were then used to determine the amount of S-GAG in the sample referring to the working curves obtained above.

RESULTS

The calibration curves obtained for each S-GAG, i.e., chondroitin 4-sulfate (Figs. 1 and 2), chondroitin 6-sulfate, and dermatan sulfate, were linear for concentrations between 0-3 μg/100 μl.

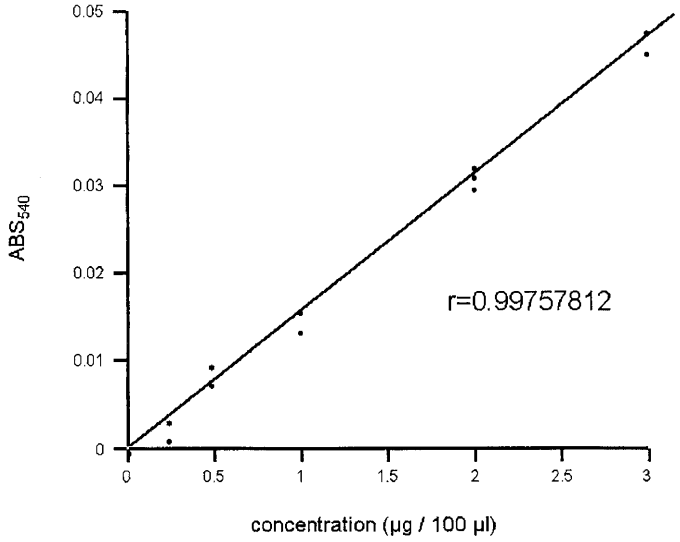


Fig. 2 Calibration curves with condroitin 4-sulfate as standard

Table 2 Amounts of S-GAG in the GCF of healthy subjects and in those with periodontal disease

	age • sex	ABS_{540}	concentration of S-GAG (μg / 100 μl)
healthy donor	25♂	0.001	0.07
	25♂	0.003	0.20
	25♀	0.000	0.00
	24♂	0.000	0.00
	23♂	0.000	0.00
cases of periodontal disease	58♂	0.006	0.40
	20♀	0.012	0.79
	63♀	0.000	0.00
	40♂	0.005	0.33
	60♂	0.006	0.40
	42♂	0.009	0.59
healthy donor (mean ± SE) :		0.05 ± 0.04 μg/100 μl, n = 5	$p < 0.05$
cases of periodontal disease (mean ± SE) :		0.42 ± 0.11 μg/100 μl, n = 6	

Other GCF components, such as glycoprotein and serum constituents, did not influence the measurement of S-GAG.

Although trace amounts of S-GAG were found in the GCF of healthy subjects (0.05 μg/100 μl ± 0.04, mean ± SE, n = 5), large amounts were found in those with periodontal disease (0.42 ± 0.11, n = 6, $p < 0.05$) (Table 2).

DISCUSSION

Embery et al.[1, 2] reported that significant amounts of S-GAG chondroitin sulfate and non-sulfated hyaluronan have been identified in the GCF of sites with severely inflamed periodontal tissues. We previously reported a very low concentration of S-GAG as chondroitin sulfate in the GCF of healthy subjects, and a significantly higher concentration in

subjects with periodontal disease[3]. We then compared the profiles of unsaturated disaccharide units obtained from GCF by HPLC analysis with those found in various tissues of the periodontium[8]. The obtained results suggested that the main source of chondroitin sulfate in GCF was mineralized tissues, particularly alveolar bone, which forms a greater part of the periodontium than cementum. Furthermore, Embery et al.[1, 2] reported that other sulfated glycosaminoglycans such as dermatan sulfate and heparan sulfate were absent from the GCF and also noted that dermatan sulfate is a predominant GAG in soft connective tissues such as gingiva and periodontal ligaments, but is not present in alveolar bone[9]; therefore, the measurement of chondroitin sulfate in GCF is important to assess the condition of the hard periodontal tissues when broken down.

Some measurements of GAG in GCF have been developed as follows: electrophoresis analysis[1], HPLC analysis[3], alcian blue dye detection system[4, 10], safranin "O" dye binding assays[11] and ELISA method to detect chondroitin sulfate isomer-linked proteoglycans[12]; however, most of the measurement methods were taken over a relatively long time or excluded interference from protein and blood substances. Although the microassay system using DMB in this study is not as sensitive as HPLC[3], its accuracy and simplicity in measuring S-GAG of GCF makes it a useful screening test for periodontal disease. The DMB procedure was originally described by Farndale et al.[5] for GAG in cartilage culture after papain treatment. The simply modified DMB method described by Moriyama et al.[7] does not even specifically determine the amount of S-GAG owing to the influence of other ingredients including high-densing protein and glycoprotein in GCF. Therefore, we added the digestion process with S-GAG-specific enzyme, chondroitinase ABC. The S-GAG data obtained in this system are quite similar to those of previous reports that significant amounts of S-GAG were identified in GCF from inflamed sites but there were few S-GAG in healthy sites. This method is therefore useful for screening S-GAG in GCF to eliminate any interference in the samples.

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