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Raised chondroitin sulphate WF6 epitope levels in gingival crevicular fluid in chronic periodontitis

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Abstract

Aim: To determine the levels of chondroitin sulphate (CS) WF6 epitope, recognized by WF6 monoclonal antibody, in gingival crevicular fluid (GCF) from different stages of periodontal disease and healthy periodontium, and to correlate those levels with clinical parameters.

Material and Methods: GCF samples, collected from 389 sites, were analysed for the WF6 epitope levels by the competitive enzyme-linked immunosorbent assay.

Results: The median WF6 epitope level was significantly higher in chronic periodontitis sites ($n = 185$) than in healthy and gingivitis sites ($n = 204$) ($p < 0.001$), whereas the median levels did not significantly differ between healthy ($n = 65$) and gingivitis sites ($n = 139$). The median level in severe periodontitis sites ($n = 60$) was significantly higher than that in moderate periodontitis sites ($n = 63$) ($p = 0.019$). Similarly, the median level in moderate periodontitis sites was significantly higher than that in slight periodontitis sites ($n = 62$) ($p = 0.001$). The WF6 epitope levels significantly correlated with probing depth ($r = 0.777$, $p = 0.001$) and loss of clinical attachment level ($r = 0.814$, $p = 0.001$).

Conclusion: Elevated CS WF6 epitope levels in GCF are associated with severity of periodontitis. The WF6 antibody may therefore be clinically applied to monitor disease severity and progression.

Key words: chondroitin sulphate; chronic periodontitis; gingival crevicular fluid; glycosaminoglycans; WF6 monoclonal antibody

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Periodontal disease is a multifactorial disease resulting in destruction of hard and soft connective tissue constituents of periodontium (Listgarten 1987). The diagnosis of periodontal disease is practically based on clinical and radiographic evaluations, which are not evident during the initial phase of disease. Therefore, the early detection of connective tissue-derived molecules from degraded periodontal tissues might lead to improved assessment of periodontal status (Armitage 2004). There has been considerable interest, in the past few decades, in applying knowledge in molecular biology to the identification of biomarkers for the early diagnosis of periodontal disease and for monitoring disease activity and progression. Various groups of researchers have reported several biomarkers representing connective tissue breakdown in periodontal disease, especially those biomarkers that derive from loss of cementum and alveolar bone (reviewed by Embery & Last 1989, Giannobile et al. 2003, Armitage 2004). Moreover, it has recently been shown that the levels of protein carbonyl, considered a marker of severe oxidative protein damage and dysfunction, are significantly higher in gingival crevicular fluid (GCF), collected from chronic perio-

Conflict of interest and source of funding statement

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dontitis sites compared with the controls (Baltacıoğlu et al. 2008).

Glycosaminoglycans (GAGs), the extracellular ground substances of connective tissues, exist in a covalently bound form of a series of hexauronate-containing heteropolysaccharides linked to a specific core protein to form high-molecular-weight aggregates, called proteoglycans (Bartold 1987). Chondroitin sulphate (CS) is the principal GAG found in cementum and alveolar bone (Bartold et al. 1988, Bartold 1990), in which chondroitin-4-sulphate (C-4-S) is predominantly present and in which chondroitin-6-sulphate (C-6-S) is detectable in much lower amounts (Waddington & Embery 1991, Waddington et al. 1998). Connective tissue breakdown by activated proteolytic enzymes in periodontal disease causes a release of GAGs from their protein core into GCF via an inflammatory exudate (Embery et al. 1982). One family of these proteolytic enzymes is matrix metalloproteinase (MMP), particularly MMP-13, whose expression is shown to be increased in gingival biopsies taken from active sites of chronic periodontitis, whereas the levels of tissue inhibitor of metalloproteinase-1 in GCF, collected from these sites, decrease (Hernández et al. 2007). Several previous studies showed the presence of C-4-S in GCF from periodontitis patients, suggesting the association between the C-4-S levels and the severity and activity of periodontal disease (Last et al. 1985, Okazaki et al. 1995, Smith et al. 1995, 1997). In addition, raised C-6-S levels have been detected in GCF from experimentally induced periodontitis in dogs (Shibutani et al. 1993).

Recently, a novel monoclonal antibody, namely WF6, which recognizes an epitope of native CS chains of embryonic shark cartilage proteoglycans, has been developed and characterized (Pothacharoen et al. 2007). This antibody has been used to examine CS levels in serum samples from patients with chronic inflammatory joint diseases, including rheumatoid arthritis and osteoarthritis (Pothacharoen et al. 2006a). Periodontitis, also, is a chronic inflammatory disorder, whose pathobiologic features are similar to those of rheumatoid arthritis. In addition, some studies have shown a relationship between rheumatoid arthritis and periodontitis (Mercado et al. 2000, 2001) and between rheumatoid arthritis and tooth loss, which represents one aspect of periodontal health (Lagervall et al. 2003, Lagervall & Jansson 2007). Consequently, because there is a relationship between rheumatoid arthritis and periodontitis, it is reasonable to apply the WF6 antibody for determining the CS WF6 epitope levels in GCF from patients with different stages of periodontal disease. The purposes of this study were to determine the levels of CS WF6 epitope in GCF obtained from healthy and diseased periodontium, and to compare the CS WF6 epitope levels with two clinical parameters of periodontal disease, namely, probing depth (PD) and loss of clinical attachment level (CAL).

Material and Methods

Patient and site selection

Twenty-two patients, aged 25–39 years (mean age = 29 ± 3.19 years) with gingivitis, and 30 patients, aged 31–68 years (mean age = 46.18 ± 7.87 years) with chronic periodontitis, were recruited from the Department of Periodontology, Faculty of Dentistry, Chiang Mai University. The research design was approved by the Ethics Committee, Faculty of Dentistry, Chiang Mai University, and written informed consent was obtained before the patients' enrolment. Exclusion criteria were: any history of systemic disease, such as diabetes mellitus, rheumatoid arthritis, or osteoarthritis; smoking; periodontal treatment or antibiotic or anti-inflammatory drug use within 3 months before sample collection; and pregnancy.

The following clinical parameters, PD, loss of CAL, and gingival index (GI) (Löe & Silness 1963), were recorded at the baseline to identify the patients' periodontal status. The PD at six sites for each tooth, to the nearest millimetre, was measured, using the PCP-UNC15 probe (Hu-Friedy, Chicago, IL, USA). The cemento-enamel junction was used as a reference for the measurement of the loss of CAL. All parameters were examined by one periodontist. The intra-examiner calibration was performed with 98% and 96% agreement for PD and loss of CAL, respectively. These two parameters were selected for the correlation with the CS WF6 epitope levels.

The patients with gingivitis manifested gingival inflammation without periodontal destruction, whereas those with chronic periodontitis were diagnosed according to the classification of the American Academy of Periodontology (AAP) 1999 (Armitage 1999). Sixty-five healthy sites (GH), whose GI score was 0, were selected as a control group from the patients with gingivitis, and 66 gingivitis sites (GG), whose GI scores were either 2 or 3, were also chosen from the patients with gingivitis (Table 1). For the patients with chronic periodontitis, 73, 62, 63, and 60 sites were selected for gingivitis (PG), slight (PS), moderate (PM), and severe (PSe) periodontitis, respectively (Table 1). Each selected site was separated by at least one tooth unit from another, in order to avoid GCF contamination. The power calculations (>90%) were carried out to determine the sample size in each group.

Sample collection

In both patients with gingivitis and those with chronic periodontitis, GCF samples were collected 1 week after periodontal recording to avoid blood contamination. Before sample collection, supragingival

Table 1. A summary of the number (*N*) of sites and the minimal (min), maximal (max), and median levels of chondroitin sulphate (CS) WF6 epitope in ng/ml in each stage of periodontal disease

Disease	Severity	*N* (sites)	CS WF6 epitope levels (ng/ml)		
			min	max	median
Gingivitis	GH	65	1.01	60.01	16.99
	GG	66	1.17	139.58	16.37
Chronic periodontitis	PG	73	1.66	114.48	12.73
	PS	62	4.34	440.60	74.36
	PM	63	23.68	926.20	303.21
	PSe	60	58.28	1072.68	381.96

GH and GG, healthy and gingivitis sites, respectively, in patients with gingivitis. PG, PS, PM, and PSe, gingivitis, slight chronic periodontitis, moderate chronic periodontitis, and severe chronic periodontitis sites, respectively, in patients with chronic periodontitis.

plaque was removed with a sterile cotton pellet. The GCF collection was conducted following the method of Cliantar & Caruana (1998). Briefly, the selected site was isolated with a cotton roll and gently air-dried. GCF samples were collected using paper strips (Periopaper, ProFlow, Amittyville, NY, USA). An analytical instrument (Periotron 8000™, Oralflow Inc., Plainview, NY, USA) was used to measure the GCF volume. The paper strips were individually kept frozen at -80°C in a microcentrifuge tube for further analysis. The GCF was recovered from the paper strip by addition of 200 μl of phosphate-buffered saline, pH 7.4, and the tube was then vigorously shaken for a few minutes. The recovery rate (approximately 98.1%) from each paper strip was determined by a dye-binding assay, using known concentrations of sulphated GAGs as standards (Ratcliffe et al. 1988).

Competitive ELISA with the WF6 monoclonal antibody

ELISA was performed as described previously (Pothacharoen et al. 2006a, b). In brief, a microtitre plate (Maxisorp®, Nunc, Roskilde, Denmark) was coated with 10 μg/ml of shark PG-A1 fraction, prepared as described in a previous report (100 μl/well) in the coating buffer (20 mM sodium carbonate buffer, pH 9.6) and stored overnight at room temperature (Kongtawelert et al. 1989). After removing the coating buffer, the uncoated area was blocked with 150 μl/well of 1% (w/v) bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in the incubating buffer [0.01 M Tris-HCl, 0.15 M sodium chloride, 0.1% (v/v) Tween-20, pH 7.4] for 60 min. at 37°C. After washing, 100 μl of the mixture, either a sample or a standard competitor (Shark PG-A1D1 fraction, whose concentrations ranged from 39.06 to 10,000 ng/ml) mixed with the WF6 monoclonal antibody (Pothacharoen et al. 2007, patent number WO 2005/118645 A1) at the dilution 1:100, was added. After incubation for 60 min. at 37°C, the plate was washed, and 100 μl/well of the IgM-specific peroxidase-conjugated anti-mouse secondary antibody (Sigma-Aldrich) at the dilution 1:2000 was added for 60 min. at 37°C. The plate was washed again, and 100 μl/well of the peroxidase substrate (Sigma-Aldrich) was added and incubated for 20 min. at 37°C. The reaction was stopped by addition of 50 μl of 4 M H_2SO_4. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader (ICN Flow, Costa Mesa, CA, USA).

Statistical analyses

The Kruskal–Wallis one-way analysis of variance test was used to compare CS WF6 epitope levels between groups both when the patient was the statistical unit, and where individual sites were the statistical unit. The Wilcoxon Mann–Whitney *U*-test ($p = 0.05$) was used to compare CS WF6 epitope levels between each pair of groups both at the patient and the site levels. The correlation between the CS WF6 epitope levels and the clinical parameters, PD and loss of CAL, was assessed by the Spearman rank order correlation ($p = 0.05$).

Results

Elevated CS WF6 epitope levels in association with the severity of periodontal disease

It was found that the median of CS WF6 epitope levels in GH and GG from patients with gingivitis, and PG from patients with chronic periodontitis were 16.99, 16.37, and 12.73 ng/ml, respectively (Table 1). In addition, there were no significant differences between the median CS WF6 epitope level in GH and that in GG ($p = 0.499$), and between the median CS WF6 epitope level in GH and that in PG ($p = 0.793$) (Fig. 1). In contrast, there was a significant difference in the median CS WF6 epitope level between non-destructive sites, including GH, GG, and PG sites (a total of 204 sites), and destructive sites, including PS, PM, and PSe sites (a total of 185 sites) ($p < 0.001$).

Among the destructive sites in chronic periodontitis patients, the median of CS WF6 epitope levels in PS, PM, and PSe were 74.36, 303.21, and 381.96 ng/ml, respectively (Table 1). It was found that the median CS WF6 epitope level in PSe was significantly higher than that in PM ($p = 0.019$), and the median CS WF6 epitope level in PM was significantly higher than that in PS ($p = 0.001$) (Fig. 1). Furthermore, the median CS WF6 epitope level in PS was significantly higher than that in PG ($p = 0.001$) (Fig. 1). Taken together, all of these results suggest that the elevated CS WF6 epitope levels in GCF can be correlated with the degree of severity in periodontal disease.

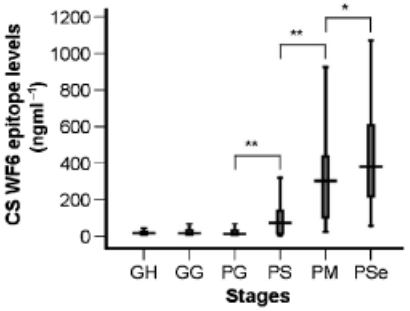

Fig. 1. The distribution of chondroitin sulphate (CS) WF6 epitope levels in different stages of periodontal disease. The *y*-axis represents the levels of CS WF6 epitope in ng/ml, and the *x*-axis represents different stages of periodontal disease. Shaded rectangles represent the values that fall between the 25th and 75th percentiles, and horizontal lines represent the median levels of CS WF6 epitope. $^{*}p < 0.05$; $^{**}p < 0.01$. GH and GG: healthy and gingivitis sites, respectively, in patients with gingivitis. PG, PS, PM, and PSe: gingivitis, slight chronic periodontitis, moderate chronic periodontitis, and severe chronic periodontitis sites, respectively, in patients with chronic periodontitis.

Interestingly, considering the patient as a unit for statistical analysis, a total of 52 patients could be classified into four groups, according to the classification of AAP 1999 (Armitage 1999), including gingivitis ($n = 22$), slight ($n = 7$), moderate ($n = 8$), and severe ($n = 15$) periodontitis groups. It was demonstrated that the median CS WF6 epitope levels were 15.80, 53.54, 75.26, and 121.60 ng/ml, respectively. Using the Kruskal–Wallis one-way analysis of variance test, significant differences were found among these four groups ($p < 0.001$). Particularly, the median CS WF6 epitope level in gingivitis group was significantly less than those in periodontitis groups ($p < 0.001$). However, the median CS WF6 epitope levels in three periodontitis groups were not significantly different ($p > 0.05$). This is due to much larger variations in the CS WF6 epitope levels observed in periodontitis groups, because there were various degrees of disease severity, ranging from gingivitis to severe periodontitis, within the same periodontitis patient, especially in the cases of severe periodontitis. This suggests that analysis of the CS WF6 epitope levels in an individual patient does not truly reflect the different degrees of disease severity at each specific site. In addition, using a multilevel approach to remove the

patient variables, it was still demonstrated that the CS WF6 epitope levels in four groups of different disease severities were significantly different ($p = 0.013$), indicating the independence of multiple sites within the same patient.

Association between the CS WF6 epitope levels and the clinical parameters

In patients with chronic periodontitis, the CS WF6 epitope levels exhibited a significant correlation with two clinical parameters, i.e. PD and loss of CAL. Using the Spearman rank-order correlation, a positive correlation was observed between the CS WF6 epitope levels and PD ($r = 0.777$, $p = 0.001$) as well as those levels and loss of CAL ($r = 0.814$, $p = 0.001$) (Figs 2 and 3, respectively). These results indicate that the levels of CS WF6 epitope, a biochemical marker tested in this study, correspond well with both clinical parameters (PD and loss of CAL), generally accepted for an evaluation of severity of periodontal disease. It is interesting to note that the median levels of CS WF6 epitope rose in accordance with increasing PD and loss of CAL (horizontal lines in Figs 2 and 3, respectively).

Discussion

The present study shows the significantly greater levels of CS WF6 epitope in GCF from destructive sites of patients with chronic periodontitis compared with those from non-destructive sites of patients with gingivitis or chronic periodontitis. The levels of CS WF6 epitope also significantly correlated with PD and loss of CAL, two gold standards commonly used to clinically evaluate periodontal disease severity. It is speculative that the increase in the CS WF6 epitope levels holds true in destructive sites from other forms of periodontitis as well. In addition, it was clearly demonstrated that the median level of CS WF6 epitope in GCF was continuously elevated in relation to the increased severity of periodontal disease status. However, while the median CS WF6 epitope levels were raised in the chronic periodontitis sites, the distribution of levels found was broad and it is interesting to note that the levels were even normal in some periodontitis sites. There were no obvious clinical signs and symptoms that distinguished the sites with high values from those with low values in this cross-sectional study. Whether these CS WF6 epitope values relate to disease activity or progression during the period of GCF collection will need to be evaluated in a more detailed longitudinal study. It is noteworthy that monitoring disease activity or progression using biochemical markers in GCF, which reflect the disease activity at the specific local site, is more suitable for periodontal disease in that a destructive change of periodontal tissue does not simultaneously occur at each individual periodontitis site. Moreover, unlike the patient's serum, the monitoring of biochemical markers in GCF would avoid other influences from underlying systemic diseases. Nevertheless, care was taken in this study to exclude from the study any patients with other related systemic disorders.

It appears that the mean age of chronic periodontitis patient group is greater than that of the control gingivitis group. This apparent limitation in recruiting age-matched control subjects in this study is acknowledged, because it is unlikely to find a sufficient number of young patients who are inflicted with chronic periodontitis or old patients who exhibit only gingivitis. It is, however, interesting to note that, in both healthy and gingivitis sites, the CS WF6 epitope levels remain low in both gingivitis and periodontitis patients, i.e. GH, GG, and PG, regardless of the subjects' age (Table 1 and Fig. 1). Consistently, in the group of periodontitis patients with varying degrees of disease severity, i.e. PG, PS, PM, PSe, the CS WF6 epitope levels in GCF are raised in accordance with the increased disease severity, but not related to the subjects' age (Table 1 and Fig. 1). This suggests that the subjects' age does not affect the WF6 epitope levels in GCF.

A few studies from our laboratory have lately shown elevated levels of CS WF6 epitope using our novel WF6 monoclonal antibody in serum samples from patients with rheumatoid arthritis and osteoarthritis (Pothacharoen et al. 2006a) and ovarian epithelial cancer (Pothacharoen et al. 2006b). Very recently, the structurally defined oligosaccharide fractions recognized by the WF6 antibody have also been characterized by an enzyme-linked immunosorbent assay using an oligosaccharide microarray (Pothacharoen et al. 2007). In this study, it was demonstrated that the WF6 antibody could detect the majority of C-6-S chain in shark cartilage, and the two reactive fractions were octasaccharides, whose sequences were DCCC and CCAD, where A, C, and D denote disaccharides with sulphation at the fourth, sixth, and both second and sixth carbon atoms, respectively. In addition, by a competitive ELISA using various types of CS chain as competitors, it was found that CS-C and -D, isolated from shark cartilage, bound the WF6 antibody with much stronger affinity than other CS chains with different sulphation patterns, including CS-A, -B, and -E, isolated from bovine tracheal cartilage, porcine skin, and squid cartilage, respectively (unpublished data). This suggests that the WF6 antibody mostly reacts with 6-sulphated disaccharide, or C-6-S, and that the WF6 epitope levels in GCF in this study, therefore, represent the C-6-S levels.

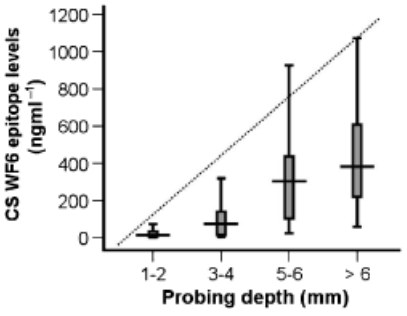

Fig. 2. The positive correlation between the chondroitin sulphate (CS) WF6 epitope levels and probing depth (PD), as shown by the projection of a diagonal dotted line. The *y*-axis represents the levels of CS WF6 epitope in ng/ml, and the *x*-axis represents PD in millimetres.

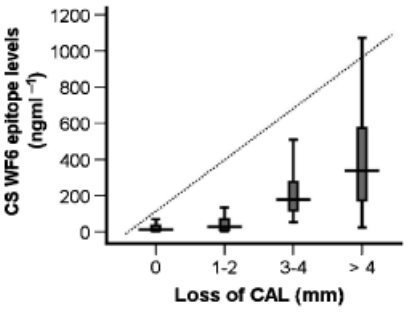

Fig. 3. The positive correlation between the chondroitin sulphate (CS) WF6 epitope levels and loss of clinical attachment levels (CAL), as shown by the projection of a diagonal dotted line. The *y*-axis represents the levels of CS WF6 epitope in ng/ml, and the *x*-axis represents loss of CAL in millimetres (mm).

Moreover, from the computer modelling, the two octasaccharide sequences share a striking similarity between the shape and distribution of the electrostatic potential, providing an explanation for their strong affinity for the WF6 antibody, although their sequences are different (Pothacharoen et al. 2007).

In the study of WF6 epitope levels in serum samples from patients with rheumatoid arthritis and osteoarthritis, we have shown that the pattern of rise in the CS WF6 epitope levels corresponds well with that in the CS 3B3(+) epitope levels (Pothacharoen et al. 2006a). The 3B3(+) epitope is a 6-sulphated terminal disaccharide on a CS chain after chondroitinase ABC digestion (Caterson et al. 1990). This implies that the elevated levels of WF6 epitope represent the levels of C-6-S derived from loss of periodontal tissue constituents, accumulated in GCF. This implication is in agreement with the results of a previous study (Shibutani et al. 1993). It is also consistent with the finding that the two reactive octasaccharide sequences contain the majority of C-6-S (Pothacharoen et al. 2007). Unlike the 3B3(+) epitope that requires prior enzymatic digestion, the native WF6 epitope on CS chains is readily detected by the WF6 monoclonal antibody without the need for prior digestion and can thus be suitable for future uses in clinical settings.

As demonstrated in this study, nanogram levels of C-6-S present in a small, limited volume of GCF samples can be detected with the more sensitive ELISA technique and the WF6 antibody in comparison with the time-consuming electrophoretic technique that requires a large volume of GCF samples (Embery et al. 1982, Last et al. 1985, 1988, Waddington et al. 1989, Smith et al. 1995). Furthermore, a greater number of samples can be concurrently analysed by the ELISA technique. Therefore, it is possible that our WF6 monoclonal antibody together with ELISA may be further developed as a chairside commercial diagnostic kit.

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Clinical Relevance

Scientific rationale for the study: There has been considerable interest in identifying biomarkers for an early diagnosis and monitoring of periodontal disease. One of these biomarkers is CS, a major constituent of alveolar bone. A recently developed antibody, which mainly recognizes C-6-S, was applied to determine the C-6-S levels in GCF from different stages of periodontal disease.
Principal findings: With this antibody, a significant increase in the C-6-S levels in periodontitis sites was shown, and the levels correlated well with the disease severity.
Practical implications: Our results suggest that this antibody may be further applied for clinical periodontology.