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Measurement of gp130 cytokines – Oncostatin M and IL-6 in gingival crevicular fluid of patients with chronic periodontitis

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Abstract

Several proinflammatory cytokines can induce periodontal tissue destruction and are thought to be useful indicators or diagnostic markers for periodontitis. Here, we aimed to investigate whether oncostatin M (OSM) was present in gingival crevicular fluid (GCF) and to clarify the correlation of GCF OSM and interleukin-6 (IL-6) levels with the severity of periodontitis. Sixty-two sites in 14 patients were divided into 4 groups based on probing depth (PD) and bleeding on probing (BOP). GCF was collected using paper strips from clinically health sites (PD $\leq$ 3 mm, CAL: 1–3 mm, without BOP, $n = 31$), mildly diseased sites (PD $\leq$ 3 mm, CAL: 3–5 mm, with BOP, $n = 11$), moderately diseased sites (PD = 4–6 mm, CAL: 5–8 mm, with BOP, $n = 11$), and severely diseased sites (PD $>$ 6 mm, CAL: 8–12 mm, with BOP, $n = 9$). IL-6 and OSM in GCF were quantified by enzyme-linked immunosorbent assay and are expressed as concentrations (pg/ml) and total amounts (pg/site). Correlations of OSM and IL-6 levels with the severity of periodontitis in all groups were determined using Spearman rank correlation (r_s). Our results showed that OSM and IL-6 were detected in most GCF samples. The total amounts of OSM and IL-6 were significantly positive correlated with severity of diseased sites (OSM: $r_s = 0.526$, $p < 0.01$; IL-6: $r_s = 0.729$, $p < 0.01$). No correlations of OSM or IL-6 concentration in GCF were found with disease severity. OSM and IL-6 levels in GCF were positively correlated to each other when expressed as either concentrations or total amounts (concentrations: $r = 0.485$, $p < 0.01$; total amounts $r = 0.490$, $p < 0.01$). In conclusion, our findings suggest that IL-6 and OSM may play a role in modulating the inflammatory cascade of chronic periodontitis.

Keywords: Gingival crevicular fluid; Interleukin-6; Oncostatin M; Periodontitis

1. Introduction

Periodontitis is an inflammatory disease characterized by the destruction of supporting tissues of teeth. Constituents of the plaque biofilm are directly or indirectly responsible for activating host inflammatory cells in periodontal tissues to secrete both catabolic cytokines and inflammatory mediators [1,2]. The cytokine profile produced by these cells is associated with the pathogenesis of periodontal diseases [2,3]. Several proinflammatory cytokines can induce periodontal tissue destruction and are secreted in the gingival crevicular fluid (GCF), including interleukin-1α (IL-1α), interleukin-1β (IL-1β), tumor necrosis factors-α (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8) [4].

IL-6 is a multifunctional cytokine produced mainly by lymphocytes, monocytes and fibroblasts. Elevated

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levels of IL-6 in blood or biological fluids have been reported in association with immunopathologies such as in tissue injury, infection and some inflammatory diseases [5,6]. Many studies have shown dramatic elevation of IL-6 levels in inflammatory periodontal lesions and IL-6 is also thought to be a useful indicator or a diagnostic marker for periodontitis [7–10].

Oncostatin M (OSM), a 28 kDa glycoprotein produced predominately by activated T lymphocytes and endotoxin stimulated macrophages, is a member of the IL-6 family which comprises IL-6, IL-11, OSM, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1) [11]. Similar to IL-6, OSM is pleiotropic and participates in diversified physiological processes such as wound healing, inflammatory response and cellular proliferation and differentiation [11,12]. There is evidence to indicate that IL-6, LIF and OSM stimulate bone resorption [13]. In addition, OSM has been detected in synovial fluids from patients with rheumatoid arthritis (RA) but not osteoarthritis [14,15] and the levels of OSM are correlated with levels of markers of the degradation of crosslinked collagen and cartilage aggrecan [15].

Modur et al. demonstrated that OSM, but not other IL-6 family members, fulfills Koch's postulates as an inflammatory mediator [16]. Furthermore, OSM alone may stimulate the production of IL-6, or act synergistically with IL-6 or TNF-α to up-regulate the production of metalloproteinases or augment IL-6 production, respectively [15,17]. This suggests that OSM may directly cause the deterioration associated with inflammatory processes or it may act in concert with other cytokines to amplify IL-6 production or contribute to the inflammatory cycle.

In the present study we hypothesized that OSM might also play a role in the pathogenesis of periodontal diseases by several lines of evidence. First of all, both OSM and IL-6 are regarded as proinflammatory cytokines and share a common receptor subunit, gp130, which is essential for their biological activity and, therefore, elicits several overlapping physiological activities including effects on hematopoietic tissues, bone, and events during inflammation [6,11,12,18]. In addition, there are analogous structures of tissues and similar pathogenesis of periodontitis and rheumatoid arthritic sites. In both conditions, persistent chronic inflammatory reactions in specialized areas with infiltration of inflammatory cells and cytokine profiles such as IL-1β, IL-6 and IL-8 are similar, supporting OSM possibly also modulating periodontal inflammation [19,20]. However, it is not yet known whether OSM exists in periodontal lesions. The purpose of this study was to evaluate OSM and IL-6 levels in GCF collected from subjects with clinically proven periodontitis subjects by the specific sandwich enzyme-linked immunosorbent assay (ELISA). Concomitantly, we tried to shed some light on the correlation between these 2 cytokines in GCF and establish a possible immunomodulatory role for OSM in the cytokine network of periodontitis.

2. Results

2.1. Expression of OSM in GCF

Levels of OSM in GCF for the 4 groups are shown in Fig. 1. OSM was detected in 22 of 31 healthy sites and 30

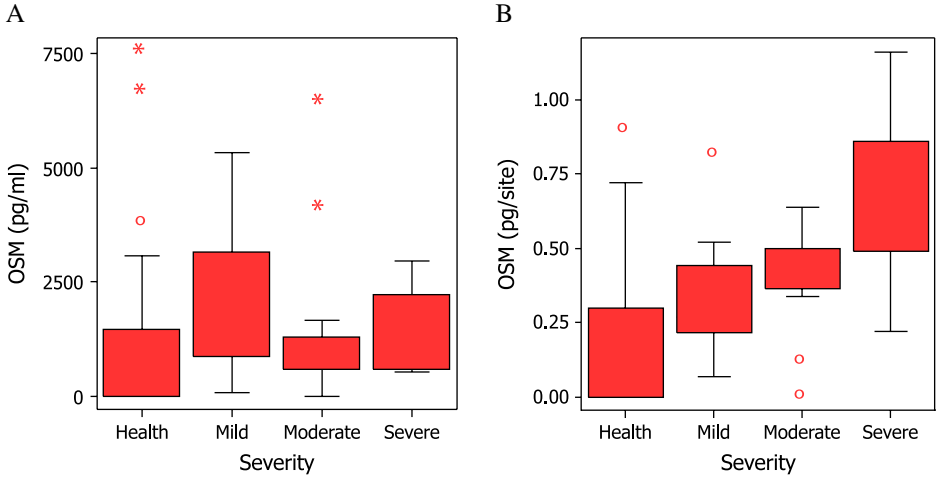

Fig. 1. OSM concentrations (A) and amounts (B) in GCF of healthy, mildly, moderately, and severely diseased sites. Concentrations of OSM showed no significance difference ($p = 0.212$) between groups, whereas there was a significant difference among the 4 groups when the data were expressed by amounts ($p < 0.01$, Kruskal–Wallis ANOVA). Compared with healthy sites, the amounts of OSM in GCF were significantly increased in severely diseased sites ($p < 0.01$, Dunn's test), whereas in the other groups of diseased sites the increase was not significant. (Box extends from 25th to 75th percentile. The line within the box is medium. The whiskers represent the range of data within 1.5 times the width of the inter-quartile range from the median value. Outlier are designated with an "o" and extreme values are designated with a "*").

of 31 diseased sites. The ranges of OSM concentrations (amounts; volume) from healthy sites and those with mild, moderate and severe periodontitis were from 68 to 7534 pg/ml (0.03–0.9 pg/site; 0.03–0.78 μl/site), 93 to 5321 pg/ml (0.7–0.81 pg/site; 0.05–0.89 μl/site), 456 to 6429 pg/ml (0.12–0.64 pg/site; 0.07–0.78 μl/site), and 521 to 2961 pg/ml (0.22–1.16 pg/site; 0.28–0.91 μl/site), respectively. There was no significant difference found among the 4 groups when the data were expressed as concentration (pg/ml) (Fig. 1A, $p = 0.212$; Kruskal–Wallis test), while significant difference was found among the groups when the data were shown as total amounts (pg/site) in GCF (Fig. 1B, $p < 0.01$; Kruskal–Wallis test).

2.2. Expression of IL-6 in GCF

Levels of IL-6 in GCF are illustrated in Fig. 2. IL-6 was detected in the GCF in 29 of 30 healthy sites and in all diseased sites. The ranges of IL-6 concentrations (amounts) from healthy sites and those with mildly, moderately and severely periodontitis were from 44 to 3924 pg/ml (0.02–0.68 pg/site), 166 to 10087 pg/ml (0.15–1.20 pg/site), 451 to 7433 pg/ml (0.25–1.40 pg/site) and 374 to 4217 pg/ml (0.38–1.31 pg/site), respectively. The ranges of GCF volume harvested with paper strips were the same as those of OSM. Total IL-6 amounts significantly differed among the 4 groups (Fig. 2B, $p < 0.001$; Kruskal–Wallis test), while no significant difference existed among the groups when the data were expressed as concentrations (Fig. 2A, $p = 0.514$; Kruskal–Wallis test).

2.3. Correlation of OSM/IL-6 in GCF and disease severity

We further examined whether the levels of these cytokines in GCF correlated with the severity of periodontitis using Spearman rank correlation (Table 1) and found the total amounts of OSM or IL-6 in GCF were positively correlated to the severity of periodontitis (OSM: $r_s = 0.526$, $p < 0.01$; IL-6: $r_s = 0.729$, $p < 0.01$). However, there was no correlation between the concentrations of both cytokines and the severity of disease sites.

Fig. 3 shows the correlation between the levels of IL-6 and OSM. The 2 cytokines were found to have a significant positive correlation with each other, when expressed as concentration (Fig. 3A, $r = 0.485$, $p < 0.01$; Spearman correlation coefficient) or total amounts (Fig. 3B. $r = 0.490$, $p < 0.01$; Spearman correlation coefficient).

3. Discussion

In this study we found that increased total amounts of OSM in GCF were positively correlated to the severity of chronic periodontitis. Although a previous study showed that OSM could promote the re-establishment of homoeostasis by cooperating with proinflammatory cytokines and suppress inflammation in murine models of rheumatoid arthritis [14], most investigations strongly suggested that OSM is a proinflammatory cytokine [15,16]. Furthermore, the levels of OSM in synovial fluid are correlated with levels of

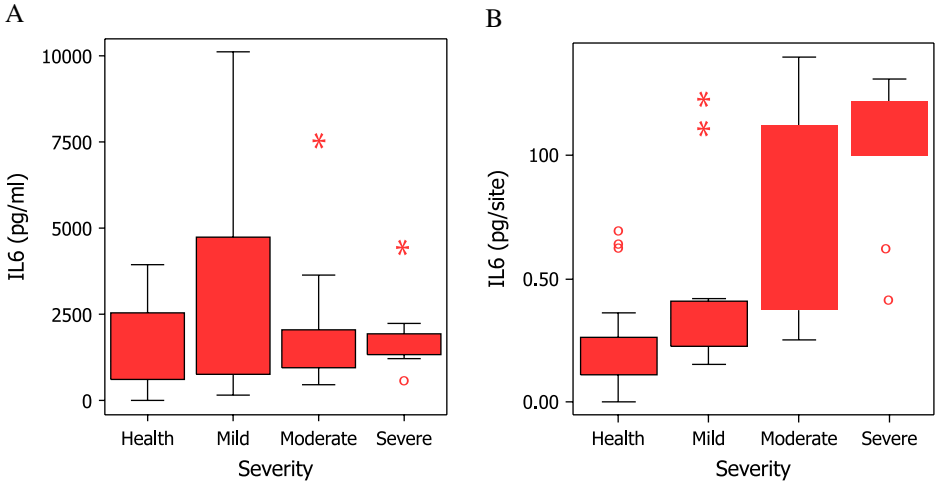

Fig. 2. IL-6 concentrations (A) and amounts (B) in GCF of healthy, mildly, moderately, and severely diseased sites. Concentrations of IL-6 showed no significance difference ($p = 0.514$), whereas there was a significant difference among the 4 groups when the data were expressed by amounts ($p < 0.001$, Kruskal–Wallis ANOVA). Compared with healthy sites, the amounts of IL-6 in GCF were significantly increased in severely diseased sites ($p < 0.01$, Dunn's test), whereas in the other groups of diseased sites the increase was not significant. (Box extends from 25th to 75th percentile. The line within the box is medium. The whiskers represent the range of data within 1.5 times the width of the inter-quartile range from the median value. Outlier are designated with an "o" and extreme values are designated with a "*").

Table 1
Correlation of the severity of periodontitis and OSM or IL-6 levels in sample sites determined by Spearman rank correlation (r_s)

Concentrations to the disease severity	r_s Value	p-Value
OSM	0.152	0.244
IL-6	0.154	0.237
Total amounts to the disease severity		
OSM	0.526	<0.01
IL-6	0.729	<0.01

degradation markers of cartilage aggrecan in RA [15]. Nevertheless, our results imply that total amounts of OSM in GCF might play certain role in the cascade of periodontal inflammation.

OSM secreted by activated monocytes is a cytokine of the interleukin-6 (IL-6) family that includes IL-6, interleukin-11 (IL-11), leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (CNTF) [22]. These entire cytokines share a common receptor subunit involved in signal transduction, gp130 [23]. IL-6, CNTF, and IL-11 initially bind to receptor subunits that recognize the respective cytokine but do not participate directly in the transduction of the signal (α subunits). On the other hand, LIF and OSM bind to receptor components (β subunits) that are also involved in the transduction of the signal. Irrespective of whether a cytokine binds to a α subunit or directly to a β subunit, our data show that OSM and IL-6 possess a positive correlation in modulating the inflammatory response of periodontal tissue, e.g. bleeding on probing.

It has been summarized that angiogenesis of soft tissue is regulated by many cytokines and growth factors such as transforming growth factor-α, transforming growth factor-β, hepatocyte growth factor, tumor necrosis factor-α (TNF-α), prostaglandin E2, angiogenin, interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) [24]. VEGF can be detected in the vascular endothelial cells, inflammatory cells, junctional, sulcular and gingival epithelium of periodontal tissue [25]. A recent report showed that OSM, present in human aortic aneurysms, is an inflammatory mediator contributing to chronic inflammation, but that the other cytokines of the IL-6 family are not involved [16]. Among the mechanisms involved in OSM-induced endothelial cell proliferation, it was observed that OSM stimulated the secretion of VEGF on human microvascular endothelial cells (HMEC-1s cell line) [26]. In our study, the diseased sites were defined by one of the criteria, bleeding on probing. The positive correlation of GCF OSM and disease severity in our investigation indicates that the existence of OSM may play certain role in regulating the bleeding events of gingival tissue; possibly a reflection of chronic inflammation via VEGF directed angiogenesis.

Several molecules present in inflammatory process have been found to stimulate bone resorption in vitro and in vivo, including interleukin-1 (IL-1), IL-6, IL-11, IL-17, OSM, leukemia inhibitory factor (LIF), tumor necrosis factor (TNF-α, TNF-β), macrophage colony-stimulating stimuli factor (M-CSF), bradykinin, thrombin and prostaglandins (PGs) [27–29]. In a study of detection of receptors for gp130 cytokines in bone marrow stromal/osteoblastic cells, it was found that stromal/osteoblastic cells are targets for the actions of all the members of the cytokine subfamily that shares the gp130 signal transducer; and suggest that different receptor repertoires may be expressed at different stages of differentiation of this lineage [30]. Two previous studies have shown that human OSM stimulates osteoclast cell formation [31,32]. Jay et al. also demonstrated that human OSM regulate IL-6 secretion, collagen secretion, and alkaline phosphatase activity in calvaria osteoblast cell cultures [33]. Richards et al. first demonstrated the unique role of OSM in the induction of osteoclast

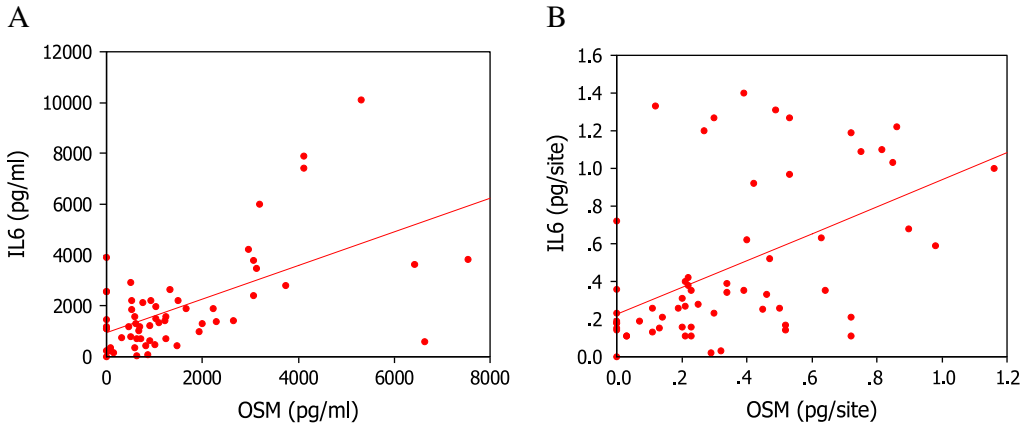

Fig. 3. Correlations between levels of OSM and IL-6 determined by Spearman rank correlation (r_s). There was a statistically positive correlation between levels of OSM and IL-6 either when the data were expressed by (A) concentrations ($r_s = 0.485$, $p < 0.01$;) or (B) total amounts ($r_s = 0.490$, $p < 0.01$).

differentiation and resorptive activity using mouse target cells in vitro [34]. These data suggest an important role of OSM in osteoporosis caused by high levels of corticosteroid. In our study, the expression of OSM and IL-6 in GCF was coincided with the severity of periodontal disease sites, especially with disease severity stratified by probing pocket depth, which is the most widely used diagnostic tool for the clinical assessment of connective tissue and bone destruction in periodontitis. It implies that, in addition to the modulation of angiogenesis, OSM may also modulate certain effect on osteoclastic bone destruction in periodontal tissue.

The multifunctional cytokine IL-6, which can be locally produced by human bronchial epithelial cells (HBECs), has been found to play a role in IL-4 dependent IgE synthesis [35]. It was also proved by Stříž et al. that Th2-type cytokines IL-4 and IL-13 affect the release of IL-6 by HBECs in response to TNF-α (inhibition) and INF-γ (augmentation). Modulation of IL-6 levels by Th2-type cytokines may play a role in allergic reactions through the IL-6 promoting effect on IL-4 mediated IgE production [36]. Inversely, IL-6 can negatively regulate the differentiation of resting CD41 T cells into effector Th1 cells by interfering with the IFN-γ receptor signaling pathway. IL-6 up-regulates suppressor of cytokine signaling 1 (SOCS1) expression in activated CD41 T cells, thereby interfering with signal transducer and activator of transcription 1 (STAT1) phosphorylation induced by interferon γ (IFN-γ) [37]. In an immunohistochemical analysis of Th1/Th1 cytokine expression of T helper cells in the pathogenesis of nifedipine-induced gingival overgrowth, we found that strong Th2-type cytokine (IL-4, IL-10, and IL-13) expression was found in inflammatory cells of severe periodontal lesions [38]. In the present investigation, it was indicated that amounts of IL-6 in GCF at sites with chronic periodontitis were significantly higher than those at healthy sites. This finding was also similar to those reports by the others [7–10,39]. We postulate that modulation of IL-6 levels by Th2-type cytokines may play a role in the immunohistologic response of periodontal disease. In addition, it has been shown that increased IL-6 levels in peripheral blood are most likely due to the enhanced production of IL-6 in locally inflamed tissues of patients with rheumatoid arthritis [40–42] and periodontitis [19,20,43] which supports the concept that the amount of IL-6 in GCF can also serve as a reference for the diagnosis of disease activity or progression of periodontal lesions.

When OSM concentrations and the severity of periodontitis were compared, increased OSM concentrations were not noted as the severity of periodontitis increased. The same phenomenon was also observed with those of GCF IL-6. Our result is similar to those reported by others that data presented by total amounts instead of concentrations are more meaningful when studying relations among GCF constituents to periodontal conditions [44–46]. One reason is that the GCF collected in paper strips is a combination of residual fluid and the inflammatory exudates present in the gingival sulcus [47]. The total volume of GCF could be dynamically affected by inflammatory exudates when local inflammatory severity is elevated. If a constituent is primarily secreted locally in GCF, increasing GCF volumes would concomitantly dilute its concentration [48]. The other reason is because we harvested GCF samples within a definitive time. The volume of GCF became a factor of dependent variance, and the data on concentrations were calculated as the values of total weight divided by volume. Therefore, to quantify a constituent in GCF, the expression of data with concentration becomes a dependent variable and has been improperly interpreted in several literatures.

The interplay between IL-6 and the other bone resorbing interleukins is complex but primarily synergistic. Our study revealed that IL-6 levels correlated positively with OSM levels. This finding is also in agreement with those of recent investigations on RA that OSM may act synergistically with IL-6 in up-regulating the production of metalloproteinases in rheumatoid joints [15]. Meanwhile, OSM can stimulate the production of IL-6 or synergize with TNF-α to augment IL-6 production in vitro [17]. In this regard, it is noteworthy that the IL-6 level was associated with changes of OSM levels in GCF of patients with chronic periodontitis.

In most tissues, inflammation is marked by a cytokine cascade with accumulation of IL-1β, IL-6, and IL-8 during progression of atherosclerotic lesions [49]. The association between periodontitis and atherosclerosis, with the primary focus on IL-6 and other related proinflammatory mediators, has been investigated in recent years. It has been suggested that OSM may play a major role in the development and complication of atherosclerosis [50]. The deleterious role of OSM is supported by the observation that OSM was identified in the atherosclerotic plaque [16]. Results of our study infer that the severity of chronic periodontitis may possibly result in increasing the systemic effect of IL-6 and OSM. These mediators may enter the circulation and affect the inflammatory activity of atherosclerotic lesions, potentially increasing the risk for cardiac or cerebrovascular events. Further investigation will be needed to approve this hypothesis by harvesting serum and GCF samples from studied subjects and analyzing the levels of atherosclerotic markers, e.g. C-reactive protein, serum amyloid A, and gp130 cytokines for the assay.

In conclusion, this study demonstrated that increased total amounts of IL-6 and OSM in GCF were positively correlated to the severity of periodontitis. To our knowledge, this is the first study to detect the presence of OSM in GCF. Yet we still do not know the exact

effect of OSM on the inflammatory mechanism of chronic periodontitis, especially on hard tissue changes. Our subsequent research will be focused on the modulatory effect of IL-6/OSM on osteoclastogenesis in periodontal disease.

4. Materials and methods

4.1. Patient and site selection

Fourteen patients (7 male patients, 30–53 y/o, average 46 years; 7 female patients, 31–64 y/o, average 53 years) were recruited from the Taipei Medical University Hospital. All of our patients were diagnosed as chronic periodontitis patients. The clinical characteristics include: >30% of sites involved with probing pocket depth ≥3 mm and simultaneous bleeding on probing; the amount of plaque free score is less than 20; subgingival calculus was frequently found; their age was usually over 30. Their general health was good and none had taken medication such as anti-inflammatory drugs, antibiotics, or contraceptives for the recent 2 weeks prior to the study. Post-menopausal and pregnancy females, or smokers [53] had also been excluded from our criteria of subject selection. No one had received any periodontal treatment within the 3 months preceding the study. All of the participants were thoroughly informed of the procedures and consequences prior to participation.

Clinical parameters which included probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP) at 6 sites on each tooth was measured with a Williams probe by the same examiner (Chen YL). Sites with BOP were defined as diseased sites [21]. Our preliminary study showed that the IL-6/OSM expression of healthy sites in periodontal healthy individuals was not significantly different from that of diseased individuals [52]. It implied that the expression of IL-6 or OSM in GCF harvested form the healthy site of patients with chronic periodontitis may be used as a local control subject under site-specific design. In the present study, sampled sites were categorized into 4 groups based on the clinical periodontal examination: clinically health sites (PD ≤ 3 mm; CAL within the range 1–3 mm; without BOP; $n = 31$), mild diseased sites (PD ≤ 3 mm; CAL within the range 3–5 mm; with BOP; $n = 11$), moderate diseased sites (PD = 4–6 mm; CAL within the range 5–8 mm; with BOP; $n = 11$), and severe diseased sites (PD > 6 mm; CAL within the range 8–12 mm; with BOP; $n = 9$).

4.2. Collection of GCF

GCF was collected from 4 to 6 sites of each patient using sterile filter paper strips (Periopaper®, Oraflow, NY, USA) in facio-proximal surfaces of single-rooted teeth by the method of Uematsu et al. [51]. Briefly, all clinically detectable and visible supragingival plaque was carefully removed without touching the gingiva to minimize plaque contamination of the paper strips. The sites under study were isolated with cotton rolls and the area around gingival crevice was gently dried with an air syringe to prevent salivary contamination. The paper strip was carefully inserted into the gingival crevice until mild resistance was felt and remained in place for 30 s. Strips of visible contamination with blood or debris were discarded. In order to eliminate the risk of evaporation, the volume of GCF in the strips was immediately measured with a GCF meter (Periotron 6000, Oraflow) that had been calibrated with serial known volumes of human serum and translated into a volume (μl) unit by Periotron Professional 3.0 software. In total, the following sites were used in our studies: 31 healthy sites, 11 mildly, 11 moderately and 9 severely diseased sites.

The sample in the strips was eluted by centrifugal filtration. Briefly, each strip was placed in a sterile Eppendorf tube and 100 μl of buffer containing 50 mM phosphate buffer, pH 7.2, 0.1 mM phenylmethylsulfonyl fluoride, 50 μg/ml each of leupeptin, antipain and pepstatin was applied to each strip for 30 min and the tube was centrifuged at 15,000*g* under 4 °C for 5 min. A second 100 μl was applied and the centrifugation repeated. After removal of the strips, supernatants were stored at −80 °C until assayed.

4.3. Determination of IL-6 and OSM

The levels of IL-6 were quantified by commercially available high sensitivity ELISA kits (Quantikine® HS; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. The sensitivity in this assay was 0.039 pg/ml, and the intra-assay and inter-assay coefficients of variation (CVs) were <7.8% and 9.6%, respectively.

The levels of OSM were determined according to the methods recommended by the manufacturer (R&D Systems). Briefly, 96 microtiter plates were first coated with monoclonal anti-human OSM antibody (4 μg/ml, R&D Systems). After blocking, the plates were incubated with samples or standards followed by polyclonal biotinylated anti-human OSM antibody (50 ng/ml, R&D Systems) and streptavidin horseradish peroxidase (Zymed Laboratories Inc., San Francisco, CA, USA). Plates were then developed with tetramethylbenzidine (Clinical Science Laboratories Inc., Mansfield, MA, USA) for 30 min at room temperature and the reaction finally stopped by the addition of 50 μl stop solution. The intra-assay CV was <3.6% and the inter-assay CV was <10.9% [14].

The optical density of each well was measured using a microplate reader set at 490 nm for the IL-6 assay and 450 nm for the OSM assay. The results were then

calculated and obtained by methods of an interpolation of pre-determined linear standard curve. Values of concentrations and total amounts were expressed as pg/ml and pg/site, respectively.

4.4. Statistical analysis

All data were entered into a computer database and analyzed by SPSS software (version 10.0, SPSS, Chicago, IL, USA). Our previous calculation showed that these data were not drawn from a normally distributed population as estimated by the Shapiro–Wilk test; therefore, the comparison between groups was performed using 2-tailed nonparametric Kruskal–Wallis test. When a significant difference was found, paired group comparisons were performed with the Dunn's test. The correlations between variables (the severity of periodontitis to OSM or IL-6 levels; OSM to IL-6 levels) were determined by Spearman rank correlation (r_s). We considered statistical difference was significant at $p < 0.01$.

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