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Identification of the osteoprotegerin/receptor activator of nuclear factor-kappa B ligand system in gingival crevicular fluid and tissue of patients with chronic periodontitis

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Background and Objective: Recent findings have suggested that osteoclastogenesis is directly regulated by receptor activator of nuclear factor-kappa B ligand (RANKL) and its decoy receptor, osteoprotegerin (OPG). However, no studies have described interactions of OPG/RANKL and the gp130 cytokine family in periodontal disease. This study aimed to identify and quantify OPG/RANKL in the gingival crevicular fluid (GCF) and connective tissue of patients with periodontitis, and to clarify possible correlations with disease severity and interleukin-6 (IL-6) cytokines.

Material and Methods: Ninety-five sites in 20 patients with generalized chronic periodontitis were divided into four groups by site based on probing depth (PD) and bleeding on probing (BOP). In periodontitis patients, GCF was obtained using sterile paper strips from clinically healthy sites (PD $\leq$ 3 mm without BOP, $n = 12$ in periodontitis subjects), mildly diseased sites (PD $\leq$ 3 mm with BOP, $n = 23$), moderately diseased sites (PD $\leq$ 4–6 mm with BOP, $n = 33$) and severely diseased sites (PD $>$ 6 mm with BOP, $n = 27$). Fourteen clinically healthy sites from four periodontally healthy individuals were used as the control group. The levels of OPG, RANKL and two gp130 cytokines – IL-6 and oncostatin M (OSM) – in the GCF were determined by an enzyme-linked immunosorbent assay (ELISA) and are expressed as total amounts (pg/site). Immunohistochemical localization of OPG- and RANKL-positive cells was also performed on gingival connective tissues harvested from patients with periodontitis (inflammatory group, $n = 8$ biopsies) and from non-diseased individuals (healthy group, $n = 8$ biopsies).

Results: GCF RANKL, but not OPG, was elevated in diseased sites of patients with periodontitis. However, the expressions of OPG and RANKL showed no correlation with disease severity ($r = 0.174$ and 0.056, respectively), but the content of RANKL in the GCF was significantly positively correlated with those of IL-6 ($r = 0.207$) and OSM ($r = 0.231$) ($p < 0.01$). Immunohistochemical staining

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showed that RANKL-positive cells were significantly distributed in the inflammatory connective tissue zone of diseased gingiva, compared with those of samples from non-diseased persons ($p < 0.01$). However, few OPG-positive cells were found in connective tissue zones of either the diseased gingiva or healthy biopsies.

Conclusion: These findings imply that in this cross-sectional study of GCF, RANKL, IL-6 and OSM were all prominent in periodontitis sites, whereas OPG was inconsistently found in a few samples of diseased sites but was undetectable in any of the control sites. The results also imply that the expression of RANKL was positively correlated with IL-6 and OSM in the GCF.

Periodontitis is an inflammatory disease characterized by destruction of the supporting tissues of the teeth. An imbalance between the plaque biofilm and the host immune system results in the overexpression of pro-inflammatory cytokines and the subsequent destruction of alveolar bone. At the molecular level, sites of bone destruction represent higher activities of osteoblasts relative to osteoclasts. However, the precise mechanism of bone destruction has not been clearly elucidated over the past few decades.

Recent findings of the discovery of the osteoprotegerin (OPG)/receptor activator of nuclear factor-kappa B ligand (RANKL)/receptor activator of nuclear factor-kappa B (RANK) system have unraveled a longstanding unexplained phenomenon in bone resorption. RANK is primarily expressed in cells of the monocyte/macrophage lineage, including osteoclastic precursors, B and T cells, dendritic cells and fibroblasts (1). RANK activation by RANKL is followed by its interaction with tumor necrosis factor (TNF) receptor-associated (TRAF) family members and activation of nuclear factor (NF)-κB, c-Fos, C-Jun N-terminal protein kinase (JNK), c-src, and the serine/threonine kinase, Akt/PKB (2,3). RANK is crucial for all calcium-tropic hormones and proresorptive cytokines to increase calcemia and multiplication of osteoclasts in bone. RANKL, a membrane-bound or soluble protein belonging to the TNF superfamily, is primarily produced in osteoblastic lineages and activated T cells, stimulates osteoclast differentiation and activation, and inhibits osteoclast apoptosis. Binding of RANKL to RANK expressed on the surfaces of osteoclasts and their precursors, promotes osteoclast differentiation and activation (4). OPG, a soluble protein that is one of the TNF receptors (TNFRs), has contrasting properties to the RANKL-mediated biological effects because it acts as an inhibitor that suppresses RANKL interaction, leading to a decrease in osteoclastogenesis (5,6).

Various cytokines, such as interleukin (IL)-1, IL-6 and IL-11, are known to modulate RANKL and OPG levels and can also be detected in the gingival crevicular fluid (GCF) (7–9). Oncostatin M (OSM), a member of the IL-6 family of cytokines, which includes IL-6, IL-11, leukemia inhibitory factor (LIF) and ciliary neurotrophic factors (CNTFs), has been demonstrated to fulfil Koch's postulates as an inflammatory mediator (10). Expression of increased OSM has also been noted in certain inflammatory bone diseases, such as rheumatoid arthritis (RA). Furthermore, OSM alone may stimulate the production of IL-6, or it may act synergistically with IL-6 or TNF-α to, respectively, up-regulate the production of metalloproteinases or augment IL-6 production (11,12). We demonstrated that increased amounts of IL-6 and OSM in the GCF were positively correlated to the severity of periodontitis (13).

In this study, we attempted to determine the profiles of OPG, RANKL, IL-6 and OSM in human GCF and aimed to establish an initial model of regulation of the OPG/RANKL system by IL-6 family cytokines in the progression of periodontal inflammation.

Materials and methods

Patient and site selection

Eleven male patients (27–53 years old; average age 46 years) and nine female patients (31–64 years old; average age 53 years) were randomly selected from patients at Taipei Medical University Hospital. Their general health was good, and none had taken medication, such as anti-inflammatory drugs, antibiotics, or contraceptives, for 2 weeks prior to the study. In addition, no patient had received any periodontal treatment within the 3 months preceding the study. All of the participants were informed, in detail, of the procedures, and signed a consent form in advance of their participation in this study. Clinical parameters included probing depth (PD) and bleeding on probing (BOP) at six sites on each tooth, which were measured using a Williams probe by the same examiner. Sites with BOP were defined as diseased sites.

Criteria for classification of periodontal severity

Patients with ≥ 8 teeth that suffered from radiographic-proven bone loss extending into the middle third of the root length or beyond, were defined as periodontitis subjects. Sites with BOP were initially defined as diseased sites from periodontitis subjects (14). Sampled sites were then categorized into four subgroups based on a clinical periodontal examination: clinically healthy sites (PD ≤ 3 mm with no evidence of BOP; $n = 12$), mildly diseased sites (PD ≤ 3 mm with BOP;

$n = 23$), moderately diseased sites (PD 4–6 mm with BOP; $n = 33$), severely diseased sites (PD > 6 mm with BOP; $n = 27$), and 14 sites selected with PD ≤ 3 mm and no evidence of BOP from the gingival sulcus of four periodontally healthy individuals (average age 30 years).

Collection of GCF

GCF was obtained using sterile filter paper strips (Periopaper®; Oraflow, New York, NY, USA) in facially proximal surfaces of single-rooted teeth. GCF from four to six sites of each patient was collected by the same operator. The area was isolated, all detectable supragingival plaque was carefully removed, and the site was gently air-dried to prevent salivary contamination. A paper strip was carefully inserted into the gingival crevice until mild resistance was felt, and it was left in place for 30 s. If a strip was contaminated with blood or debris, then it was discarded. The fluid collected on the strip was immediately transferred to a GCF meter (Periotron 8000; Oraflow), which had been calibrated with known serial volumes of human serum, and was translated into volume (μl) units by PERIOTRON PROFESSIONAL 3.0 software (Oraflow).

In order to completely extract the sample from the paper, we eluted the fluid by the method of centrifugation with portions of buffers. Briefly, each strip was placed in a sterile Eppendorf tube and incubated in 200 μl of a phosphate buffer solution (50 mM; pH 7.2) for 30 min. Thereafter, each tube was centrifuged at 15,000 *g* and 4°C for 10 min. After removal of the strips using centrifugal filtration, supernatants were stored at −80°C until assayed (15).

Quantification of OPG, RANKL, IL-6 and OSM in GCF

The contents of OPG and RANKL in the samples were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (Biomedica Medizinprodukte, Wien, Austria). The concentration of IL-6 was quantified by commercially available highly sensitive ELISA kits (Quantikine® HS; R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The concentration of OSM was determined according to methods recommended by the manufacturer (R & D Systems). Briefly, 96-well microtiter plates were first coated with monoclonal antihuman OSM immunoglobulin (4 μg/ml; R & D Systems). After blocking, the plates were incubated with samples or standards followed by polyclonal biotinylated antihuman OSM immunoglobulin (50 ng/ml; R & D Systems) and streptavidin horseradish peroxidase (Zymed Laboratories, San Francisco, CA, USA). Plates were then developed with tetramethylbenzidine (Clinical Science Laboratories, Mansfield, MA, USA) for 30 min at room temperature, and stop solution was eventually added to terminate the reaction. The intra-assay coefficient of variance (CV) was < 3.6%, and the interassay CV was < 10.8%.

The optical density of each well was measured using a spectrophotometer set to 490 nm for the IL-6 assay and 450 nm for the OSM, OPG and RANKL assays. All samples and standards were assessed in duplicate. Data were then calculated and obtained by methods of interpolation of a predetermined standard curve. Values of total amounts are expressed as pg/site.

Identification of OPG- and RANKL-positive cells in gingival connective tissue

Sample collection Biopsies from inflamed tissues (comprising those from the mild, moderate and severe disease groups, each $n = 8$) were taken during periodontal flap surgery under local anesthesia. Gingival samples ($n = 8$) of the healthy group were harvested from wedge tissues of individuals whose periodontal status was clinically healthy and who had agreed to undergo a crown-lengthening procedure for prosthodontic purposes.

Sample preparation and sectioning Samples were fixed with 3.5% formaldehyde in 0.1 M phosphate buffer at 4°C. After 24 h of fixation, all specimens were washed in 0.05 M phosphate-buffered saline (PBS) and then dehydrated and embedded in paraffin. Nine specimens for each of the OPG and RANKL groups were selected from every five sections of 5-μm-thick serial sections for immunohistochemical staining. Sections were mounted on slides that had previously been coated with 3-aminopropyltriethoxysilan (APES; Sigma, St Louis, MO, USA) to avoid detachment of tissue samples, and were treated for specific OPG and RANKL immunohistochemical staining.

Immunohistochemical staining Sections were deparaffinized, rehydrated and pretreated with Tris-HCl buffer for 25–30 min. Afterwards, these samples were incubated with a 1 : 50 dilution of goat antihuman OPG (sc-21039; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and with a 1 : 50 dilution of goat antihuman RANKL (sc-7627; Santa Cruz Biotechnology) overnight at 4°C. Both antibodies were diluted in 1% bovine serum albumin (BSA) before use. Endogenous streptavidin-biotin (Vector Laboratories, Burlingame, CA, USA) was used as the blocking agent. Immunodetection was performed using a Vectastain Universal Quick kit (Vector Laboratories) and diaminobenzidine (DAB) as the substrate–chromogen system (Dako, Carpentaria, CA, USA).

Cell counting A grid scan was used to determine the 0.1 × 0.1-mm square field for cell counting, and five areas were randomly chosen in connective tissue area (× 200) of each specimen.

Statistical analysis

All statistical analyses were computerized and analyzed by SPSS software (version 10.0; SPSS, Chicago, IL, USA). It was found that these data were not drawn from a normally distributed population, as estimated by the Shapiro-Wilk test. Therefore, site cytokine levels were compared among groups using the Kruskal–Wallis one-way analysis of variance and between each pair of groups using the

Mann–Whitney *U*-sum rank test. Strong correlations between variables were determined by Spearman rank correlation coefficients. We considered a statistical correlation to be significant at $p < 0.05$ for IL-6/RANKL and OSM/RANKL, and at $p < 0.01$ for IL-6/OSM. As for the immunohistochemical analysis, data were analyzed using the Mann–Whitney *U*-Wilcoxon test to compare the intensities of OPG- and RANKL-positive cells in gingival connective tissues of diseased sites and control non-diseased sites. The mean data were calculated as the percentage of positive cells relative to the total number of cells counted in each field.

Results

Amounts of OPG and RANKL

Box-and-whisker plots for the content values of GCF OPG and RANKL are illustrated in Fig. 1. OPG was detected in 60 of 83 diseased sites and in nine of 12 clinically healthy sites in diseased subjects, but not in any healthy subject. RANKL was detected in all groups, except for one control site. The ranges of OPG and RANKL content in healthy individuals were, respectively, 9.67–40.73 and 0–45.16 pg/site, while values from the healthy sites of patients with periodontitis were 0–7.75 and 27.16–72.66 pg/site, from sites with mild periodontitis were 0–29.05 and 22.02–280.89 pg/site, with moderate periodontitis were 0–27.71 and 27.69–285.75 pg/site, and with severe periodontitis were 0–27.35 and 0–285.75 pg/site, respectively. In diseased subjects, OPG showed no significant differences in intergroup comparisons, while RANKL was markedly increased in moderately and severely diseased sites compared with healthy sites (Mann–Whitney *U* rank sum test, $p < 0.05$).

Amounts of IL-6 and OSM

The levels of IL-6 and OSM in GCF for the five groups are shown in Fig. 2. In periodontitis subjects, IL-6 and OSM were detected in amounts ranging from 0 to 3.51 and 0–4.77 pg/site in healthy sites, and from 0 to 4.79 and 0–5.10 pg/site in mildly diseased sites, 0.37–4.84 and 0.85–6.60 pg/site in moderately diseased sites, and 1.05–7.25 and 1.31–7.80 pg/site in severely diseased sites, respectively. The ranges of IL-6 and OSM content values were measured at 0–2.14 and 0–2.05 pg/site, respectively, in the healthy individual group. In diseased subjects, there was a statistically significant intergroup comparison (Kruskal–Wallis analysis of variance, $p < 0.01$). IL-6 and OSM showed positive correlations with increased disease severity (Table 1, both $p < 0.01$).

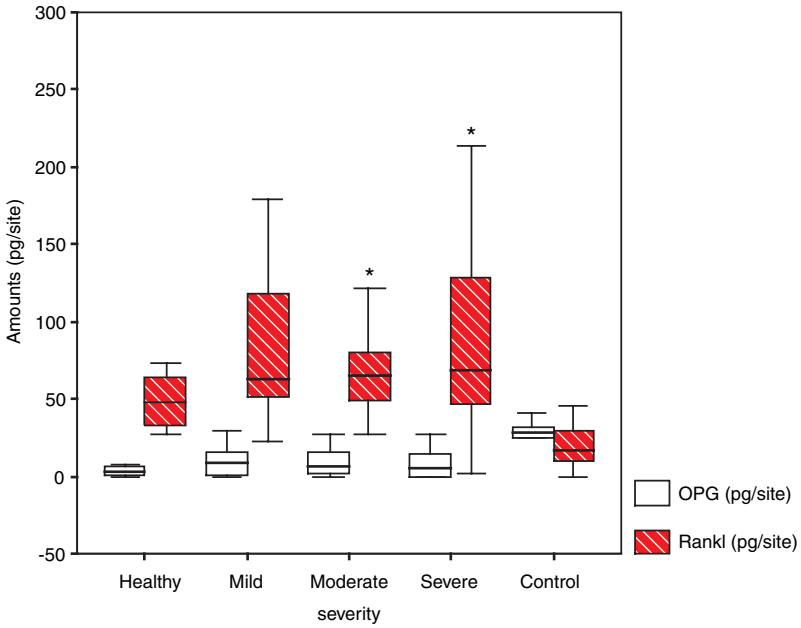

Fig. 1. Levels of osteoprotegerin (OPG) and the receptor activator of nuclear factor-kappa B ligand (RANKL) in gingival crevicular fluid (GCF) obtained from diseased and healthy subjects. In diseased subjects, the concentrations of OPG and RANKL were not significantly different among groups, as calculated by the Kruskal–Wallis one-way analysis of variance ($p = 0.275$ and 0.109, respectively). *$p < 0.05$ as compared with healthy sites of diseased individuals, determined by the Mann–Whitney *U*-Wilcoxon test. (The box extends from the 25th to the 75th percentiles. The line within the box is the median. Whiskers extend to the largest and smallest values within 1.5 box lengths.).

Comparison of IL-6/OSM and OPG/RANKL levels of healthy sites from both healthy and diseased subjects

No statistically significant differences were observed in the content values of IL-6 and OSM between healthy sites of healthy subjects and healthy sites of diseased subjects (Fig. 2), but Fig. 1 shows a statistically significant difference in the expression of RANKL between these two groups (Mann–Whitney *U*-Wilcoxon test, $p < 0.01$), but not of OPG.

Correlations of disease severity, OPG, RANKL, IL-6 and OSM in diseased subjects

To determine whether the levels of OPG, RANKL, IL-6 and OSM in GCF were correlated with the severity of periodontitis and whether a correlation existed between cytokines, the correlation coefficients were analyzed using Spearman's rank test (Table 1). Total amount of OSM and IL-6, but not of OPG or RANKL, in GCF were positively correlated with the severity of periodontitis ($p < 0.01$). In addition, the Pg/site of IL-6/RANKL, OSM/RANKL and IL-6/OSM were found to have significantly positive correlations with each other ($p < 0.05$, < 0.05 and < 0.01, respectively). Differences existed in the intensities of OPG- and RANKL-positive cells between diseased sites and control, healthy sites.

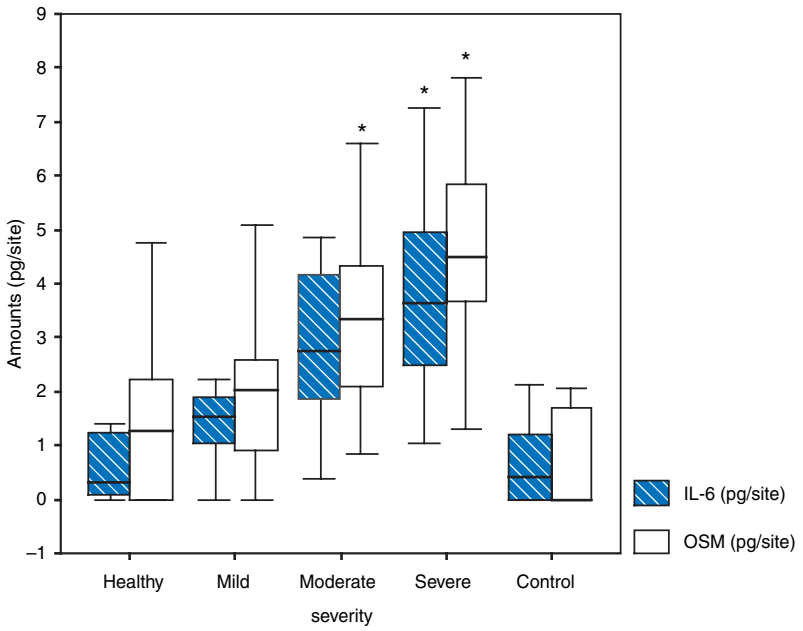

Fig. 2. Levels of oncostatin M (OSM) and interleukin-6 (IL-6) in the gingival crevicular fluid (GCF) from diseased and healthy subjects. In diseased subjects, the amount of IL-6 and OSM showed significant differences among groups, as calculated by the Kruskal–Wallis one-way analysis of variance ($p < 0.001$). *$p < 0.01$ compared with healthy sites of diseased individuals, determined by the Mann–Whitney *U*-Wilcoxon test. (The box extends from the 25th to the 75th percentiles. The line within the box is the median. Whiskers extend to the largest and smallest values within 1.5 box lengths.).

Table 1. Correlation coefficients for disease severity, interleukin-6 (IL-6), oncostatin M (OSM), receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG)

Parameter	Disease severity	IL-6	OSM	RANKL	OPG
IL-6	0.615**	–	–	–	–
OSM	0.615**	0.412**	–	–	–
RANKL	0.174	0.207*	0.231*	–	–
OPG	0.056	0.063	0.146	−0.094	–

*Correlation is significant at the $p < 0.05$ level (Spearman rank correlation coefficients).
**Correlation is significant at the $p < 0.01$ level (Spearman rank correlation coefficients).

Table 2. Mann–Whitney *U*-test statistics for calculation of receptor activator of nuclear factor-kappa B ligand (RANKL)-positive cells (%)

Group	*n*	Minimum	Maximum	Mean	SD
Healthy	8	0.80	18.72	7.88	6.87
Inflamed	8	33.54	79.91	53.70*	15.48

*Asymmetrically significant (two-tailed), $p < 0.01$.
SD, standard deviation.

Immunohistochemical staining results showed no differences in the distribution of OPG-positive cells in either diseased or healthy connective tissue sites. OPG-positive cells were rarely scattered in the diffuse zone of inflammation in the gingival connective tissue of diseased samples. However, RANKL-positive cells were widely distributed in the gingival connective tissue of patients with chronic periodontitis [mean ± standard deviation (SD), 53.70% ± 15.48%]. The Mann–Whitney *U*-test showed a significant difference in the RANKL-positive cell percentage between diseased sites of patients with chronic periodontitis and those of healthy individuals (Table 2, $p < 0.01$).

Discussion

Our data suggest that from the evidence of the positive correlations among GCF RANKL and IL-6/OSM, the crosstalk between members of the TNF family and gp130 family might play a certain role in the progression of periodontal disease. Numerous factors are known to increase bone resorption. Richards *et al.* first demonstrated the unique role of OSM in the induction of osteoclast differentiation and resorptive activity using mouse target cells (16). Human OSM may also regulate IL-6 secretion, collagen secretion and alkaline phosphatase activity in calvaria osteoblast cell cultures (17). OSM and IL-6 are known to stimulate mesenchymal progenitor differentiation toward the osteoblastic lineage and are also potent anti-apoptotic agents on osteoblastic cells (18). However, the main sources of IL-6 in bone are osteoblastic cells and stromal cells, not osteoclastic cells (19). IL-6 stimulates osteoclast activity and bone resorption by an indirect mechanism, increasing the interactions between osteoblasts and osteoclasts. Functional studies have indicated that receptors on osteoblasts, but not on osteoclasts, are required in order for IL-6 to stimulate osteoclast differentiation (20). In addition, osteoblasts express both subunits of the IL-6 receptor, gp130 and IL-6α receptors, and the receptor complex is activated by IL-6 (21). Therefore, the first action of IL-6 is to stimulate osteoblastic production of downstream effectors, which subsequently activate osteoclasts, among which the RANKL is of concern. The RANKL produced by osteoblasts can act in either a paracrine manner to activate osteoclast activity directly, or in an autocrine manner, similar to that of IL-6, to stimulate osteoblasts to produce further RANKL, which directly activates osteoclasts.

Numerous cytokines, such as IL-1α, IL-1β, TNF-α, IL-6 and IL-8, are known to modulate the OPG/RANKL/RANK system and increase bone resorption (22). In a culture study using recombinant RANKL or OPG to enhance or decrease cytokine production in mouse RANKL-deficient T cells or normal T cells, it was shown that OPG and RANKL have no direct effect on the cytokine production of T cells (23). However, it was convincing that IL-6 may modulate the OPG/RANKL/RANK system through directly increasing RANKL expression in osteoblasts and stromal cells (24,25). Conversely, IL-6 has little effect on the levels of OPG mRNA in cultures of human periodontal ligament cells (4).Our study showed that the total content of RANKL, but not of OPG, was significantly correlated with both OSM and IL-6 in the GCF of diseased sites. This implies that in the cascade of periodontal inflammation, human T cells and monocyte lineages can synthesize and secrete large amounts of OSM and IL-6 in response to bacterial products. Those members of the gp130 family of cytokines appeared to play a key role in regulating periodontal bone resorption by acting on both osteoblasts and osteoclasts through autocrine IL-6 and paracrine RANKL regulation.

The up-regulation of RANKL, and the down-regulation of OPG, have been observed in various inflammatory bone diseases, such as osteoporosis, RA, periodontal diseases and multiple myelomas (1,8,9). OPG acts as a decoy receptor to compensate for the RANK, for RANKL binding and inhibits osteoclast maturation and activation both *in vitro* and *in vivo* (5,6); nevertheless, multiple functions of the RANKL include promotion of osteoclast differentiation, activation of osteoclasts and inhibition of osteoclast apoptosis (26,27). It was speculated that expression of the RANKL coincided with the net interaction of RANKL and OPG. A semiquantitative image analysis demonstrated that significantly higher levels of RANKL protein were expressed in periodontal lesions. Conversely, OPG protein was significantly lower in periodontitis tissue (28). Another semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) study showed that the level of RANKL mRNA was highest in advanced periodontitis. In contrast, the level of OPG mRNA in both advanced and moderate periodontitis was lower than that in the healthy group (8). Our data, on the OPG content of diseased sites, demonstrated that 25% of the samples had undetectable levels in the OPG assay. The GCF OPG of all sample sites of healthy individuals was also below the detection level. The immunohistochemical analysis also proved that unlike OPG, RANKL-positive cells were widely distributed in the inflammatory tissue of diseased gingiva. This is in accordance with information from the above literature that up-regulation of the RANKL and down-regulation of OPG may be considered to be the master mechanisms modulating local bone destruction in periodontitis.

Dhore *et al.* demonstrated significant RANKL and OPG immunoreactivities in non-diseased vessel walls and in early atherosclerotic lesions in human tissues, whereas in advanced atherosclerosis lesions, only the RANKL was detected in the extracellular matrix surrounding calcium deposits (29). A recent study found that OPG levels in serum were ≈30% greater in women with diabetes than in those without diabetes in an elderly female group, and a significant correlation of elevated OPG serum levels with the prevalence of cardiovascular mortality was also noted (30). Interestingly, OPG-deficient mice exhibit both a decrease in bone density and calcification of vessels (31). It is possible that increased serum levels of OPG act as a protective factor counteracting disease progression. OPG and RANKL might be involved not only in regulating osteoclastogenesis but also atherosclerosis. In the present study, a significant difference in the expression of the GCF RANKL was found between healthy sites of diseased subjects and control sites of healthy individuals (Fig. 1). This implies that the inflammatory response of periodontal lesions may exert certain systemic effects on the cardiovascular system through periodontal RANKL secretion. However, when the cytokine expression of healthy sites in control individuals was compared with that in diseased individuals, our results showed no significant differences in the expression of IL-6 and OSM between these two groups (Fig. 2). This implies that the expression of IL-6 and OSM in the GCF of diseased sites may present only local infectious factors in periodontal lesions. We conjectured that although the gp130 cytokine family only exerted a local response in periodontal lesions, modulation of IL-6 and OSM in atherosclerosis may still occur indirectly via the up-regulation of RANKL expression. In conclusion, in this cross-sectional study of GCF in periodontal disease, IL-6, OSM and the RANKL were prominent in periodontitis sites, whereas OPG was not detectable in some diseased sites and healthy control sites. IL-6 and OSM may activate up-regulation of the RANKL, leading to the sequence of destruction of the periodontium. Although the content of IL-6, OSM, RANKL and OPG in GCF were investigated in our study, our conclusions were limited to the GCF. For the purpose of explaining the roles of IL-6 and OSM in the pathogenesis of periodontitis and the RANKL/OPG/RANK system, further *in vitro* cell culture studies and *in vivo* animal model studies are needed.

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