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Gingival crevicular fluid and serum leptin: their relationship to periodontal health and disease

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Abstract

Background & Aims: Leptin is a pleiotrophic hormone produced by adipose tissue and it plays an important role in protection of the host from inflammation and infection. The purpose of this study is to determine the presence of leptin in gingival crevicular fluid (GCF) and serum samples and to find out their association, if any.

Methods: Forty two subjects were selected based on their body mass index and were divided into three groups of 14 each; healthy (Group I), chronic gingivitis (Group II) and chronic periodontitis (Group III). GCF samples (by microcapillary pipettes) and serum samples (by venipuncture) were collected to estimate the levels of leptin using enzyme linked immunosorbent assay kit.

Results: The highest mean leptin concentration in GCF was obtained for Group I (2658 pg/ml) and the least for Group III (1312 pg/ml). In contrast, the lowest serum leptin concentration was obtained for the Group I (8783 pg/ml), and the highest for Group III (12082 pg/ml). This suggests a negative correlation of GCF leptin concentration and a positive correlation of serum leptin concentration as the clinical attachment level progresses ($p < 0.05$).

Conclusion: These results suggest that greater the periodontal destruction, lesser is the GCF leptin concentration and greater the serum leptin concentration.

Key words: cardiovascular disease; gingiva and immunity; gingival crevicular fluid; leptin; periodontal disease; serum

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Gingivitis and periodontitis are primarily bacterial infections caused by a diverse group of micro-organisms (Loe et al. 1965). Though microorganisms are implicated as the aetiologic agent that brings about the inflammatory lesion, it is the chemical mediators of inflammation that play a pivotal role in the loss of connective tissue, as well as supporting alveolar bone (Genco 1992).

Leptin, 16 kDa non-glycosylated peptide hormone, has been classified as a cytokine as it shows structural similarities to the long chain helical cytokine family [interleukin (IL)-6] (Zhang et al. 1994). It has been suggested that leptin orchestrates the host response to infectious and inflammatory stimuli as it stimulates the immune system by enhancing pro-inflammatory cytokine production and phagocytosis by macrophages (Ahima & Flier 2000). Therefore, during infection and inflammation, leptin expression is modulated in a manner similar to the cytokine response to infection and injury.

In our recent study (Karthikeyan & Pradeep 2006), the leptin levels in gingival crevicular fluid (GCF) in periodontal health and disease was estimated. The results suggested that leptin levels decreased progressively in GCF as periodontal disease progressed. The above findings have led to the suggestion that clinically healthy tissue is ''armed'' with pro-inflammatory host components to aid in maintaining an infection-free periodontium. Further, the plasma levels of leptin have been reported to increase in infections (Sarraf et al. 1997). The elevated plasma leptin concentration has been suggested as a risk factor for cardiovascular disease by promoting atherosclerosis and enhancing calcification of arterial walls (Tartaglia et al. 1995, Kang et al. 2000, Parhami et al. 2001).

However, till date, leptin concentration in GCF and serum in periodontal health and disease has not been explored.

Hence, the aim of the present study is twofold: to assess the concentration of human leptin in GCF and serum and to

Conflict of interest and source of funding statement

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find out their association, if any, in periodontal health and disease.

Material and Methods

The study group consisted of 42 subjects whose age (30–39 years) and sex (21 males and 21 females)-matched and fell in the normal Body Mass Index (BMI) chart of WHO (WHO Expert Consultation 2004). attended the outpatient section, Department of Periodontics, Government Dental College and Hospital, Bangalore, and were selected randomly for the study. Patients with aggressive periodontitis, gross oral pathology, pregnant women, habits of smoking, alcoholism, anomalies of the immune system and those who had taken medication affecting periodontal status or had received periodontal therapy in the preceding 6 months were excluded from the study. The protocol was clearly explained to all the patients and informed consent was obtained from all recruits. The Ethics Committee, Government Dental College, Rajiv Gandhi University of Health Sciences, Bangalore, approved the study protocol.

Clinical examination

For each patient, modified gingival index (MGI) (Lobene et al. 1986), Ramfjord Periodontal Disease Index (PDI) (Ramfjord 1959), probing depth (PD) and clinical attachment level (CAL) values were recorded. All clinical parameters were measured with a Williams probe calibrated in millimetres. Six sites are examined for each tooth; mesiobuccal, buccal, distobuccal, mesiolingual, lingual and distolingual. One calibrated examiner monitored the patients and collected the clinical reports.

Based on the MGI, PDI and radiograph evidence of bone loss, subjects were categorized into three groups. Group I (healthy) consisted of 14 subjects with clinically healthy periodontium and with no loss of clinical attachment. Group II (chronic gingivitis) consisted of 14 subjects who showed clinical signs of gingival inflammation without any attachment loss. Group III (chronic periodontitis) consisted of 14 subjects who had signs of clinical inflammation and loss of attachment in excess of 1 mm and PD $\geqslant$4 mm at three to four sites in more than four teeth in each quadrant.

Collection of GCF

GCF samples were obtained from one site in each patient of all the recruits. In Group II, the site that showed the highest MGI and PDI score was selected. Similarly in Group III, the site which showed the highest MGI, PDI and CAL (range 1–4 mm) score was selected for GCF sampling. The test site selected for sampling was isolated with cotton roll, and supragingival plaque was removed with a curette (Hu Friedy, Gracey, IL, USA), without touching marginal gingiva. The crevicular site was then dried gently with an air syringe. Samples of GCF were obtained before probing into the site by placing white colour-coded 1–5 μl calibrated volumetric microcapillary pipettes (Sigma Aldrich Chemicals Company Limited. St. Louis, MO, USA). From each test site, a standardized volume of 1 μl was collected using the calibration on micropipette and placing the tip of the pipette extracrevicularly (unstimulated). The test site that did not express any volume of GCF and micropipette, which were contaminated with blood and saliva, were excluded from the study. The GCF collected was immediately transferred to a plastic vial and stored at -70°C till the time of assay.

Collection of serum

Two millilitre of blood was collected from the antecubital fossa by venipuncture using a 20-gauge needle with 2 ml syringes and immediately transferred to the laboratory. The blood sample was allowed to clot at room temperature and, after one hour, serum was extracted from blood by centrifuging at 3000 *g* for 5 min. The extracted serum was immediately transferred to a plastic vial and stored at -70°C till the time of assay.

Leptin analysis

The samples were assayed for leptin levels using commercially available enzyme-linked immunosorbent assay (ELISA). The assays were conducted according to the manufacturer's instructions. Highly sensitive ELISA kit (Biosource International Inc., Camarillo, CA, USA) was used to detect the leptin levels in the sample. Each plate was checked before using to ensure the calibration curve measured leptin standards (0–1000 pg/ml) within the stated limits of the assay. The samples were run in duplicates. The kit made use of biotin conjugate and human leptin antibody. Absorbance of the substrate colour reaction was read on ELISA reader (Molecular Dynamics, Sunnyvale, CA, USA) using 405 nm as primary wavelength. The total leptin was determined in picograms (pg), and the calculation of the concentration in each sample was performed by dividing the amount of leptin by the volume of sample (pg/ml).

Statistical analysis

Kruskal–Wallis analysis and Mann–Whitney *U*-test was carried out for a comparison of leptin levels between the groups. Using Spearman's rank correlation coefficient, the relation between leptin concentration and the clinical parameters were analysed (SPSS Inc. version 10, Chicago, IL, USA). *p*-values <0.05 were considered statistically significant.

Results

Clinical parameter

The levels of leptin in GCF and serum were determined and their correlations with the clinical parameters were analysed. As shown in Table 1, there were significant differences in the clinical parameter values when the groups were compared with each other. Further, the subjects in Group III had high levels of MGI and PDI scores as compared with Groups I and II.

GCF and serum leptin levels

All the samples in each group tested positive for leptin assay. The mean leptin concentration in GCF and serum obtained from the sampling sites are presented in Table 2. The highest mean leptin concentration in GCF was obtained in Group I (2658 pg/ml) and the least mean leptin concentration in Group III (1312 pg/ml). The mean

Table 1. Clinical parameters in sampling sites (mean $\pm$ SD)

Variable	No. of samples	MGI	PDI
Group I	14	0.36 ± 0.15	
Group II	14	1.82 ± 0.42	2.02 ± 0.50
Group III	14	2.25 ± 0.30	4.21 ± 0.39

MGI, modified gingival index; PDI, periodontal disease index.

Table 2. Concentrations of leptin in gingival crevicular fluid (GCF) and serum (mean ± SD)

Variable	No. of sites	GCF (pg/ml)	Serum (pg/ml)
Group I	14	2658 ± 339	8783 ± 1748
Group II	14	1669 ± 349	10554 ± 1825
Group III	14	1312 ± 215	12082 ± 1815

Table 3. Values obtained with the non-parametric Kruskal–Wallis test to check the equality of means for the three groups in gingival crevicular fluid and serum samples

Variable	Mean ranks (GCF)	Mean ranks (Serum)	*p* Value
Group I	35.4	11.8	<0.05
Group II	18.6	22.4	<0.05
Group III	10.4	30.3	<0.05

GCF, gingival crevicular fluid.

Table 4. Mann–Whitney *U* test or pair-wise comparison of mean leptin levels in gingival crevicular fluid and serum samples

Variable	Mean rank (GCF leptin)	Mean rank (Serum leptin)	Inference
Group I vs. Group II	21.4 and 7.6	10.3 and 18.7	Significant
Group I vs. Group III	21.5 and 7.5	9.0 and 20.0	Significant
Group II vs. Group III	18.6 and 10.4	11.2 and 17.8	Significant

GCF, gingival crevicular fluid.

concentration of Group II (1669 pg/ml) was intermediate between the highest and lowest values. In contrast, the highest mean leptin concentration in serum was obtained for Group III (120821 pg/ml) and the least mean leptin concentration was obtained for Group I (8783 pg/ml). The mean concentration of the Group II (10,554 pg/ml) were intermediate between the highest and lowest values. The analysis of variance showed that the difference in levels of leptin concentration between these groups were statistically significant at $p<0.05$ as shown in Table 3. Table 4 shows multiple comparisons using Mann–Whitney *U*-test, which was carried out to find out which pair or pairs differ significantly at 5% level of significance. When Groups I and II, I and III, II and III were compared, the differences in their means were statistically significant. The results suggest that leptin levels decreased progressively in GCF and increased progressively in serum from health to periodontitis.

Correlation

Table 5, shows Spearman's rank correlation between the clinical parameters, GCF and serum leptin levels. A significant negative correlation between the GCF and serum leptin concentration was observed. Further, a negative correlation was found for GCF leptin concentration and a positive correlation for the serum leptin concentration for the disease severity measures i.e., MGI, (in Group I) and MGI, PDI (in Groups II and III), MGI, PDI and CAL (in Group III). This suggests that leptin levels in both GCF and serum are inversely proportional to each other.

When Kruskal–Wallis test was done to compare the mean leptin concentration in GCF and serum at different CAL levels, there was a statistically significant reduction of GCF leptin levels and increase in serum leptin levels as CAL progressed (Table 6).

Discussion

Leptin, is a 16 kDa non-glycosylated peptide hormone mainly produced by adipocytes (Zhang et al. 1994), and in minor quantities by placenta (Hassink et al. 1997), T cell (Sanna et al. 2003), osteoblast (Gordeladze et al. 2002) and gastric epithelium (Bado et al. 1998).

Recently, increasing evidence is mounting on the immunomodulatory role of leptin and link between nutritional status and immune function. It has been shown that it enhances the body's immune mechanism by activation of monocytes and macrophage functions like phagocytosis and cytokine production by macrophages, chemotaxis and oxidative species production by stimulated PMNs (Caldefie-Chezet et al. 2001), development/maintenance of NK-cell (Zhao et al. 2003) and shifting T-cell responses towards T-helper cell (Th1) cytokine type (IL-2 and INF-γ) and inhibit Th2 cell (Lord et al. 1998), induction of expression and secretion of IL-1 receptor antagonist (IL-1Ra) by human monocytes in vitro by 1.4-fold, suggesting an anti-inflammatory action of leptin (Gabay et al. 2001).

The leptin receptor is homologous to gp-130, the signalling transducing subunit of IL-6 family cytokines, which are important mediators of the acute phase reactant protein (APR). Leptin expression is regulated during the APR. Leptin levels are acutely increased by inflammatory and infectious stimuli such as lipopolysaccharide (LPS) and cytokines. (Grunfeld et al. 1996) Therefore, during infection and inflammation, leptin expression is modulated in a manner similar to the cytokine response to infection and injury (Faggioni et al. 2001). Thus, the overall increase in leptin during infection and inflammation indicates that leptin is part of the immune response and host defence mechanisms.

Further, leptin is involved in at least two different bone-controlling mechanisms, a direct stimulatory effect on bone growth (osteoblast proliferation, differentiation and prolonging the life span of human primary osteoblasts by inhibiting apoptosis) (Gordeladze et al. 2002), and/or an indirect suppressive effect on bone through the hypothalamus (Takeda et al. 2002). The local environment may provide bone cells with signals favouring constant growth, whereas the central negative signal determines the density and length.

Leptin concentrations in healthy and diseased gingiva were evaluated by Johnson & Serio (2001) to define its possible role in periodontal disease progression. Healthy (non-haemorrhagic gingiva adjacent to a $\leqslant 3$ mm gingival sulcus) and inflamed gingiva (haemorrhagic gingiva adjacent to a >3 mm periodontal pocket) was assessed within

Table 5. Results of Spearman's Rank Correlation (r) test to compare GCF, Serum, MGI and CAL within the groups

Pairs of variables	GCF to serum (r)	GCF to MGI (r)	Serum to MGI (r)	GCF to PDI (r)	Serum to PDI (r)	GCF to CAL (r)	Serum to CAL (r)
Group I	− 1.000*	− 1.000*	1.000*	–	–	–	–
Group II	− 0.987*	− 0.996*	0.982*	− 0.996*	0.987*	–	–
Group III	− 0.999*	− 0.995*	0.992*	− 0.700*	0.705*	− 0.964*	0.958*

*Indicates there is significant correlation between pairs of variables.
GCF, gingival crevicular fluid; MGI, modified gingival index; PDI, periodontal disease index ; CAL, clinical attachment loss.

Table 6. Results of Kruskal–Wallis test comparing mean concentration of GCF and serum leptin with respect to CAL (mean ± SD)

Study group	CAL	No. of sites	GCF (pg/ml)	Serum (pg/ml)
Group 3	1	3	1590 ± 80*	9788 ± 538*
	2	5	1399 ± 83*	11549 ± 738*
	3	3	1166 ± 28*	12874 ± 142*
	4	3	1036 ± 22*	14471 ± 1381*

*$p < 0.05$ significant.
GCF, gingival crevicular fluid; CAL, clinical attachment loss.

solubilized gingival biopsies using ELISA method. Leptin concentrations were found to be highest within gingiva adjacent to ⩽3 mm sulcus. This study showed that human leptin is present within healthy and marginally inflamed gingiva and decreases in concentration as the adjacent PD increases. Yesim Bozkurt et al. (2006) evaluated GCF leptin levels and the influence of long-term heavy smoking on GCF leptin levels in patients with chronic periodontitis. They found that GCF leptin levels were significantly lower in smokers than non-smokers. This shows that smoking may dysregulate leptin levels. Recently, we assessed the concentration of human leptin levels in GCF from healthy periodontium, chronic gingivitis and chronic periodontitis patients. Results showed that there is a strong negative correlation between the GCF leptin concentration and periodontal disease progression (Karthikeyan & Pradeep 2006).

However, in all these previous studies, no attempts were made to estimate serum leptin concentration so as to know it's correlation with the gingival leptin concentration. Hence, the present study undertaken is the first of its kind to assess the concentration of human leptin levels in GCF and serum from healthy periodontium, gingivitis and chronic periodontitis patients. Further, these concentrations were correlated to gain insight into its possible role in the initiation and progression of periodontal disease.

The results of the current study showed a strong negative correlation between the GCF leptin concentration and periodontal disease progression and our results are in accordance with the study done by Johnson & Serio (2001), who also showed that leptin concentration is correlated negatively with the probing pocket depth. During sepsis, a negative correlation between leptin concentration and patient survival was found (Torpy et al. 1998) a similar state to that within gingival tissues in periodontal disease progression. The higher concentration of GCF leptin levels seen in periodontal health shows that it could be protective to gingival tissues. The exact mechanism of how it is protective is not known; it can also be coincidental rather than casual. Moreover, the mechanism underlying its decline in periodontal disease progression is not known. It is speculated that during inflammation leptin may be used up as a substrate or may be an artefact. Further investigation has to be carried out to probe deeper into this aspect.

Conversely, there was a rise in serum leptin concentration as periodontal disease progressed. This could be attributed to two mechanisms. First, from gingiva, as during gingival inflammation there is expansion of vascular network caused by vascular endothelial growth factor, which possibly increases the net rate of leptin removal from the gingival tissue and could raise serum leptin levels. Thus, gingiva, in addition to adipose tissue, could be a source of circulating leptin in patients with periodontal disease (Johnson & Serio 2001). Secondly, it could be a body defence mechanism to counteract periodontal inflammation as leptin is a part of the immune response and host defence mechanism (Arnalich et al. 1999).

Moreover, it has been suggested that rise in serum leptin concentration acts as a risk factor for cardiovascular diseases as it promotes atherosclerosis by enhancing platelet aggregation (Nakata et al. 1999), neovascularization of atherogenic plaque (Kang et al. 2000), induction of oxidative stress in arterial wall (Beltowski et al. 2003) and also acceleration of hepatic degradation and clearance of high-density lipoprotein apoproteins (Silver et al. 1999). Further, it induces calcification of arterial walls, thereby losing elasticity (distensibility), making them stiffer (Parhami et al. 2001). This reduces blood flow and increases heart exertion, thereby increasing the workload on the heart. The rise in serum leptin levels above 10,000 pg/ml is considered as a risk factor for cardiovascular disease (Michael et al. 2001, Yamagishi et al. 2001). In our study, as the periodontal disease progressed, there was a rise in serum leptin concentration on an average up to 12,082 pg/ml. Based on this, it may be hypothesized that a rise in serum leptin concentration due to periodontal disease could act as one of the risk factors for cardiovascular disease. However, longitudinal studies are required to confirm this possibility.

In the light of our results, we suggest that greater the periodontal destruction, lesser is the GCF leptin concentration and greater the serum leptin concentration. This observation extends our knowledge that leptin may have a protective role in periodontal health (Johnson & Serio 2001, Karthikeyan & Pradeep 2006, Yesim Bozkurt et al. 2006). Further, increased serum leptin concentration is a known risk factor for cardiovascular disease (Parhami et al. 2001, Yamagishi et al. 2001). This

increase due to periodontal disease progression, as seen in our study, indicates that the latter may raise the risk of developing cardiovascular disease. Prospective studies with a larger sample size are essential to consider the rise in serum leptin as a risk factor for cardiovascular disease.

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Clinical Relevance

Scientific rationale for the study: Leptin is a part of host defence mechanism, but little is known about it's role in periodontal disease progression. This study aimed to estimate leptin levels in GCF and serum in periodontal health and disease.

Principal findings: As periodontal disease progressed, GCF leptin concentration decreased and serum leptin concentration increased.

Practical implications: It appears that the protective effect of leptin on periodontal health is lost and the simultaneous rise in serum leptin levels may act as one of the risk factors for cardiovascular disease. This potentially significant observation could open a new era in the field of periodontal medicine.