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Interleukin-4, a T-helper 2 cell cytokine, is associated with the remission of periodontal disease

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Background and Objectives: Interleukin-4 (IL-4), secreted mainly by T-helper 2 cells, is a key cytokine for the growth and proliferation of B lymphocytes. Previous studies have proved that IL-4 has an anti-inflammatory effect owing to its efficient inhibition of the production of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α), IL-1α, IL-1β, IL-6 and IL-8 by monocytes/macrophages. The aim of the present study was to assess the relation between clinical parameters and concentrations of IL-4 within gingival crevicular fluid from inflamed gingiva and periodontitis sites and, subsequently, after treatment of the periodontitis sites.

Material and Methods: A total of 60 subjects were divided into three groups based on gingival index (GI), pocket probing depth and clinical attachment loss (CAL): healthy (group 1), gingivitis (group 2) and chronic periodontitis (group 3). A fourth group (group 4) consisted of 20 subjects from group 3, 6–8 weeks after treatment (i.e. scaling and root planing). Gingival crevicular fluid samples collected from each patient were quantified for IL-4 using the enzymatic immunometric assay.

Results: The highest mean concentration of IL-4 was obtained for group 1 (99.39 ± 49.33 pg/mL) and the lowest mean concentration of IL-4 was obtained for group 3 (15.78 ± 21.92 pg/mL). The mean IL-4 concentrations for group 2 (64.34 ± 39.56 pg/mL) and group 4 (68.92 ± 42.85 pg/mL) were intermediate between the levels in healthy subjects and periodontitis subjects.

Conclusion: The mean concentration of IL-4 decreased from periodontal health to disease. Thus, we suggest that type 2 helper T cell cytokine, as represented by IL-4, was associated with the remission or improvement of periodontal disease.

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Chronic periodontitis is characterised by an interaction between dental plaque antigen and components of the host defence system (1); therefore, periodontitis is considered to be an immunological disease with several unique immunological features, including elevated cellular and humoral immune responses (2).

Advanced cellular immunological techniques have led to a new concept that cytokines produced by several eukaryotic cells are essential for the development, growth, proliferation and differentiation of immunocompetent cells (2). One such immunocompetent cell, the lymphocyte, in particular the T-helper 1 (Th-1) and 2 cells (Th-2), produces an array of cytokines essential for B cell responses and for the maintenance of tissue homeostasis (3). Destruction of this homeostasis in the cytokine communication network would lead to the development of immunological disease such as periodontitis (2,4). The interleukins are important cytokines

primarily involved in communication between leukocytes and other cells such as epithelial cells, fibroblasts and endothelial cells (5).

Interleukin-4 (IL-4) is a key cytokine for the growth and proliferation of B lymphocytes. It is secreted mainly by T-helper 2 cells and also by macrophages, monocytes, mast cells, basophils and non-immune cells such as fibroblasts and endothelial cells (2). Owing to its stimulatory effect on B lymphocytes, IL-4 is called a 'T-cell-derived B cell stimulatory factor' (6).

The anti-inflammatory effect of IL-4 results partly from its efficient inhibition of the production of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1α, IL-1β, IL-6 and IL-8 by monocytes/macrophages (7,8). Additionally, it significantly inhibits the production of other proinflmmatory mediators such as reactive oxygen species, reactive nitrogen species and prostaglandins in monocytes/macrophages. However, IL-4 enhances the production of IL-1 receptor antagonist, which possesses an effective anti-inflammatory properties (7,9–12).

It was found that IL-4 could induce apoptosis of macrophages (13). In periodontitis sites where IL-4 levels were found to be too low, there was persistent accumulation of activated macrophages, leading to increased destruction of the periodontium (13). It has been hypothesized that localized absence of IL-4 at the site of gingival inflammation plays a fundamental role in the progression of gingivitis to periodontitis (14). An inverse relationship has been reported between gingival crevicular fluid IL-4 levels and periodontal status (15); IL-4 was higher in the periodontally healthy group, but very low in the periodontally diseased group. Similarly, Kabashima *et al*. (16) reported lack of IL-4 in gingival crevicular fluid from severe inflammatory sites.

Interleukin-4 plays an important role in the regulation of the immunoinflammatory response. The aim of the present study was to further investigate the role of IL-4 in periodontal disease by assessing its gingival crevicular fluid levels in subjects in an Indian population with healthy periodontium, gingivitis or periodontitis and to further determine the influence of non-surgical periodontal treatment on the IL-4 levels.

Material and methods

Sixty subjects, who were age-matched (29–42 years) and sex-matched (30 males and 30 females), attending the out patient section at the Department of Periodontics, Government Dental College and Hospital, Bangalore, Karnataka, India, were selected randomly for the study. Written informed consent was obtained from those who agreed to participate voluntarily, and ethical approval for the study was obtained from the institution's ethical committee. Patients with aggressive periodontitis, diabetes, bleeding disorders, gross oral pathology, habits of smoking or alcoholism, who had taken medication affecting periodontal status or had received periodontal therapy in the preceding 6 months, were excluded from the study.

Each subject underwent a full-mouth periodontal probing and charting, along with periapical radiographs using the long-cone technique. Radiographic bone loss was recorded dichotomously (presence or absence) to differentiate chronic periodontitis patients from other groups. Furthermore, no delineation was attempted within the chronic periodontitis group based on the extent of alveolar bone loss. Based on the gingival index (GI; 17), pocket probing depth, clinical attachment loss (CAL) and radiographic evidence of bone loss, subjects were categorized into three groups. Group 1 (healthy) consisted of 20 subjects with clinically healthy periodontium, with GI = 0, CAL = 0 mm and pocket probing depth ≤ 3 mm. Group 2 (gingivitis) consisted of 20 subjects who showed clinical signs of gingival inflammation, GI > 1, without any attachment loss, pocket probing depth ≤ 3 mm. Group 3 (chronic periodontitis) consisted of 20 subjects who had signs of clinical inflammation, GI > 1, attachment loss with radiographic evidence of bone loss and pocket probing depth ≥ 4 mm. Patients with chronic periodontitis (group 3) were treated with a non-surgical approach, i.e. scaling and root planing, and gingival crevicular fluid samples were collected from the same sites 6–8 weeks after treatment to constitute group 4 (after-treatment group).

Site selection and fluid collection

All the clinical and radiological examinations, group allocation and sampling site selection were performed by one examiner (A.R.P.), and the samples were collected on the next day by a second examiner (Y.R.). This was done to prevent contamination of the gingival crevicular fluid with blood, which is associated with probing of inflamed sites. Only one site per subject was selected as a sampling site. In the healthy group, to ensure an adequate volume, gingival crevicular fluid was pooled from multiple sites with no inflammatory signs. In gingivitis patients, the site with the greatest clinical signs of inflammation, i.e. redness, bleeding on probing and oedema in the absence of CAL, was selected. In chronic periodontitis patients, sites with > 2 mm CAL were identified using a Williams's graduated periodontal probe, and the site showing the greatest clinical signs of inflammation and highest CAL along with radiographic confirmation of bone loss was selected for sampling. On the sample collection day, after gently drying the area with a blast of air, supragingival plaque was removed without touching the marginal gingiva. The area was isolated using cotton rolls to prevent contamination by saliva, and gingival crevicular fluid was collected by placing a microcapillary pipette at the entrance of the gingival sulcus, gently touching the marginal gingiva. From each test site, a standardized volume of 1 μL was collected using the calibration on the white colour-coded 1–5 μL calibrated volumetric microcapillary pipette (Sigma-Aldrich, St Louis, MO, USA) with an extracrevicular approach (without stimulating the gingiva). Each sample collection was allotted a maximum of 10 min, and

some test sites in the healthy group which did not express any volume of gingival crevicular fluid within the allotted time, were excluded from the study. Furthermore, micropipettes which were suspected of being contaminated with blood and saliva were excluded from the study. The gingival crevicular fluid collected was immediately transferred to an airtight plastic vial and stored at −70°C until the time of assay.

Interleukin-4 assay

Interleukin-4 was estimated using a Human IL-4 ELISA KIT (catalog no: 850.020.096, Diaclone research, BESANCON, Cedex, France). The gingival crevicular fluid samples were expelled from the microcapillary pipettes with a jet of air using a blower provided with the pipettes and by further flushing them by a fixed amount of the diluent.

After appropriate dilution of gingival crevicular fluid samples, the samples and the standards (provided with the kit) were added to the appropriate wells in the microtitre plate. The plate was then covered and incubated for 2 h at room temperature. The plate was later washed thrice using 400 μL of wash solution. Fifty microlitres of diluted biotinylated anti-IL-4 was added to the wells. The plate was sealed again and incubated at room temperature for 1 h, following which it was washed again. One hundred microlitres of Streptavidin–horseradish peroxidase solution (provided with the kit) was added to all the wells and incubated at room temperature for 30 min. The microtitre plate was then emptied and washed thrice. One hundred microlitres of ready-to-use chromogen was added and the plate incubated in the dark for 20 min. Then 50 μL of stop solution was added to every well and the plate was read immediately by placing it in a spectrophotometric reader (ELISA reader, Molecular Dynamics, Sunnyvale, CA, USA).

The concentrations of IL-4 in the tested samples were estimated using the reference calibrated standard curve, plotted using the optical density values of the standards (provided with the kit). All the samples and standards were assayed in duplicate as suggested by the manufacturer.

Statistical analysis

All data were analysed using a software program (SYSTAT, version 11, and SIGMASTAT; SYSTAT Software, Point Richmond, CA, USA). The Kruskal–Wallis test, Mann–Whitney *U*-test and Wilcoxon signed-rank test were carried out to compare IL-4 levels between groups. Spearman's rank correlation was used for a comparison of IL-4 levels between the groups and the clinical parameters.

Results

Of the 20 samples in each group, three gingival crevicular fluid samples in group 1 tested negative for IL-4 (i.e. IL-4 could not be detected). Similarly, five samples in group 2 and 12 samples in group 3 tested negative for IL-4, while the number of samples negative for IL-4 decreased from 12 in group 3 to five in group 4, i.e. after non-surgical periodontal therapy. The highest mean concentration of IL-4 was obtained in group 1 (99.39 ± 49.33 pg/mL) and the lowest for group 3 (15.78 ± 21.92 pg/mL). The mean IL-4 concentration for group 2 (64.34 ± 39.56 pg/mL) was intermediate between the healthy and the periodontitis levels, and the mean IL-4 concentration for group 4 (68.92 ± 42.85 pg/mL) was between the healthy and gingivitis levels. The mean concentration and range of IL-4 levels in all the groups, together with standard deviation, are shown in Table 1. The Kruskal–Wallis test, which was used to compare the means, showed that the difference in levels of IL-4 amongst these groups were statistically significant at $p < 0.001$, as shown in Table 2. The results suggest that IL-4 levels decreased progressively in gingival crevicular fluid from healthy to periodontitis subjects. The Mann–Whitney *U*-test was carried out for multiple comparisons to explore which pair or pairs differed significantly at the 5% level of significance. The results showed that the differences were statistically significant between groups 1 and 2, groups 1 and 3, and groups 2 and 3 ($p < 0.005$; Table 2). The Wilcoxon signed-ranks test was carried out to compare the median IL-4 concentration between groups 3 and 4, and showed a significant difference, suggesting a substantial increase in IL-4 levels after treatment (Table 3).

Table 1. Descriptive statistics of the study population showing mean, standard deviation, median and range for the age, GI, CAL, PDI and IL-4 concentrations

	Groups	Age (years)	GI	CAL (mm)	PDI	IL-4 (pg/mL)
Group 1 (n = 20)	Mean ± SD	35.25 ± 3.393	0	0	0	99.39 ± 49.33
	Median	5.00	0	0	0	106.50
	Range (min, max)	(30, 42)	–	–	–	(0, 184)
Group 2 (n = 20)	Mean ± SD	32.80 ± 2.89	1.80 ± 0.42	0	2.10 ± 0.55	64.34 ± 39.56
	Median	32.00	1.80	0	2	82.30
	Range (min, max)	(29, 39)	(1.10, 2.60)	–	(1, 3)	(0, 112.7)
Group 3 (n = 20)	Mean ± SD	33.95 ± 3.07	2.17 ± 0.51	3.30 ± 1.13	6.30 ± 1.26	15.78 ± 21.92
	Median	33.50	2.30	3	6	0
	Range (min, max)	(30, 40)	(1, 2.9)	(2, 6)	(5, 9)	(0, 60.6)
Group 4 (n = 20)	Mean ± SD	33.95 ± 3.07	0.31 ± 0.13	2.15 ± 1.04	3.15 ± 0.75	68.92 ± 42.85
	Median	33.50	0.32	2	3	85.85
	Range (min, max)	(30, 40)	(0.1, 0.6)	(1, 5)	(2, 4)	(0, 119.8)

Table 2. Comparison of IL-4 concentrations among the groups using the Kruskal–Wallis test and the Mann–Whitney *U*-test

Kruskal–Wallis test			Mann–Whitney *U*-test		
Groups	Mean rank	*p*-value	Groups compared	Mean rank	*p*-value
Group 1 ($n = 20$)	60.13	$p < 0.005$*	Group 1 vs. group 2	27.1 and 13.9	$p < 0.005$**
Group 2 ($n = 20$)	38.92		Group 1 vs. group 3	28.4 and 12.60	
Group 3 ($n = 20$)	19.60		Group 2 vs. group 3	27.0 and 14.0	

* Significant difference amongst the groups.
** Significant difference between the paired groups compared.

Table 3. Wilcoxon signed-rank test to compare mean IL-4 concentrations between groups 3 and 4

Groups	*Z*-value	*p*-value
Group 3 Group 4	−3.408	0.001*

* Significant.

Spearman's rank correlation test to look for any correlation between the gingival crevicular fluid IL-4 concentration and clinical parameters showed a significant negative correlation between them, except for GI in groups 2 and 4 (Table 4).

Discussion

The host defence system and the cells of the periodontium are all linked by complex processes in which soluble mediators and cytokines co-ordinate tissue turnover, inflammatory processes and immune response.

In the early stage of periodontal disease, there is an increasing leukocyte infiltrate dominated by T and B lymphocytes with characteristics of both Th-1 and Th-2 cells. Subsequently, B cells dominate the lesion, with some T cells, macrophages and neutrophils, all of which secrete cytokines when exposed to bacterial products. Cytokines such as IL-1β and Tumor necrosis factor-α (TNF-α) mediate these immuno-inflammatory rections. In contrast, cytokines secreted by Th-2 cells, such as IL-4, have anti-inflammatory effects such as downregulation of IL-1 and Tumor necrosis factor (TNF) gene expression in human peripheral monocytes (8) and induction of apoptosis of activated macrophages (13).

Giannopoulou *et al*. (15) reported an inverse relationship between gingival crevicular fluid IL-4 levels and periodontal status; IL-4 was higher in the periodontally healthy group, but very low in the periodontal disease group. This is in agreement with Kabashima *et al*. (16), who reported lack of IL-4 in gingival crevicular fluid from severe inflammatory sites. Similar results were also obtained by Shapira *et al*. (14), who reported that IL-4 could not be detected in periodontitis lesions compared to gingivitis lesions. All these findings suggest that localized absence of IL-4 in gingival tissue might lead to the development of periodontitis from gingivitis.

The present study is the first to analyse the levels of IL-4 in gingival crevicular fluid in periodontal health and disease. The results of our study are in accord with those of Kabashima *et al*. (16) and Giannopoulou *et al*. (15). The number of gingival crevicular fluid samples testing positive for IL-4 decreased with the severity of periodontal disease and the mean concentration decreased from periodontal health to disease. Thus, our results support the previously proposed hypothesis (14) that localized absence of IL-4 could possibly be associated with increased periodontal destruction, and reinforce the protective role of IL-4 in periodontium.

In the present study, the use of a commercially available, sensitive ELISA kit to quantify IL-4 from selected sites allowed us to detect the smallest amount of IL-4 in the samples (sensitivity of 0.5 pg/mL). Furthermore, gingival crevicular fluid was collected using microcapillary pipettes to avoid non-specific attachment of the analyte, which is seen with filter paper fibers (18). The only disadvantage of this method is the possibility of trauma to the marginal gingiva, and utmost care was taken to avoid this during gingival crevicular fluid collection. The microcapillary pipette was placed at the entrance of the gingival crevice until it gently touched the gingival margin, and the collection time was restricted to not more than 10 min. Furthermore, loss of gingival crevicular fluid due to sticking of the sample to the capillary walls was avoided by flushing the capillary with a fixed amount of diluent.

In the present study, we have made an attempt to control the influence of a variable such as age on the levels of IL-4. The selected subjects in all three groups fell in the age range of 29–42 years, thus minimizing the influence of this variable. The high variability of IL-4 concentration in each group could be due to different stages of disease process at the time of collection of the gingival crevicular fluid samples.

We also studied the effect of scaling and root planing on the gingival crevicular fluid levels of IL-4 in subjects from group 3. The number of samples positive for IL-4 and its mean concentration increased after scaling and root planing (group 4). Thus, Th-2 cytokine, as represented by IL-4, was associated with the remission or improvement of periodontal inflammation. The effect of initial periodontal therapy on IL-4 levels was further confirmed when the

Table 4. Spearman's rank correlation coefficient

Groups	IL-4 and GI	IL-4 and pocket probing depth	IL-4 and CAL
Group 2	+0.193*	+0.601*	–
Group 3	−0.659*	−0.782*	−0.650*
Group 4	−0.245*	−0.739*	−0.751*

*Indicates if the correlation co-efficient value is between 0 and 0.5, there is a weak correlation; between 0.5 and 1, there is a strong correlation and if the value is 1, there is perfect correlation. The '+' or '–' sign indicates that the correlation is positive or negative respectively.

Wilcoxon signed-rank test yielded a significant difference before and after treatment ($p < 0.005$). Also, the increase in IL-4 levels correlated with the decrease in clinical measures of periodontal disease, viz. GI, PDI scores and CAL levels, as shown by the Spearman rank correlation test.

The findings of the present study indicate that the concentration of IL-4 in gingival crevicular fluid of chronic periodontitis subjects was lower compared to that of healthy subjects. Although the reason for this localized reduction in not known, these data seem to highlight the protective role of IL-4. It has been shown that IL-4 induces apoptosis in lipopolysaccharide- or IL-1-stimulated human monocytes and macrophages from peripheral blood (13). Lack of IL-4 may result in a breakdown of the regulation of immune function and enhanced macrophage survival in the inflammatory lesion. Subsequent macrophage accumulation may result in an enhanced stimulatory effect of bacterial lipopolysaccharide, and a subsequent elevated secretion of bone-resorbing mediators, such as prostaglandin E_2, TNF-α and IL-1β. Macrophage secretory products may themselves act to amplify localized tissue destruction (14).

Similarly, Yamamoto *et al.* (13) demonstrated that addition of exogenous human recombinant IL-4 to gingival macrophage cultures leads to cell death by apoptosis. These findings suggest that topical application of recombinant IL-4 may inhibit the persistence of macrophages in chronic periodontitis, which could then lead to decreased inflammation. This might provide a novel, non-invasive treatment modality for the management of chronic periodontitis. Such a promising field needs to be explored in future research.

Thus, we suggest that Th-2 cytokine, as represented by IL-4, was associated with the remission or improvement of periodontal disease, and its potential role in the management of periodontitis merits further investigation.

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