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Crevicular fluid endothelin-1 levels in periodontal health and disease

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Background and Objective: Endothelin-1 is a 21-amino-acid peptide with multifunctional regulation. Initial research indicated that endothelin-1 levels in the gingival crevicular fluid from patients with chronic periodontitis were higher than those in the gingival crevicular fluid from healthy subjects. The aim of the present study was to assess the relationship between the clinical parameters and the concentrations of endothelin-1 within the gingival crevicular fluid from inflamed gingiva and periodontitis sites and, subsequently, after the treatment of periodontitis sites.

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

Results: Endothelin-1 was not detected in any sample from any of the study groups.

Conclusion: The results showed that all the gingival crevicular fluid samples were negative for the endothelin-1 molecule. Therefore, endothelin-1 cannot be considered as a potential biomarker of periodontal disease progression.

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Endothelin belongs to a family of cysteine-rich peptides that has been the subject of much interest in the past two decades. Endothelin was first identified by Highsmith (1) and was subsequently isolated, sequenced, cloned and named by Yanagisawa in 1988 (2,3). Many diverse and overlapping functions of these peptides have since implicated endothelins in both homeostatic mechanisms as well as in disease. The endothelins are a family of 21-amino-acid peptides of which there are three distinct isoforms (endothelin-1, endothelin-2 and endothelin-3) (3). Endothelin-1 is the most abundant isoform and has been the best characterized. The endothelin-1 gene encodes a 212-amino-acid precursor peptide, preproendothelin, which is cleaved by a neutral endopeptidase to form a 38-amino-acid proendothelin-1 called big endothelin-1. This big endothelin-1 is further cleaved by endothelin-converting enzyme to endothelin-1 (4). Currently, two distinct human endothelin receptors are known: endothelin-A (endothelin-Ar) receptor and endothelin-B (endothelin-Br) receptor (3).

In previous studies, it has been shown that endothelin-1 has multifunctional regulation that may be relevant for the regulation of vascular tone (5), blood pressure (6) and sodium and water homeostasis (7). Recently, it has also been reported that endothelin-1 is associated with some inflammatory diseases (8). Endothelin-1 is induced in various tissues during inflammatory processes, such as gastric mucosal inflammation (9), intraocular

inflammation (10) and airway inflammation (11). In addition, it has been indicated that some inflammatory cytokines, such as interleukin-1α, interleukin-1β, interleukin-8 and tumor necrosis factor-α, up-regulate the production of endothelin-1 (8).

Periodontitis is a chronic inflammatory state that is caused by the host response to various bacterial infections. It has recently been found that *Porphyromonas gingivalis*, one of the major pathogens responsible for causing periodontitis (12), stimulates the expression of endothelin-1, with up-regulation of inflammatory cytokines and intercellular adhesion molecule-1, in the bronchial epithelial cell line HEp-2 (13). Similar induction of endothelin-1 by *P. gingivalis* in KB cells has been demonstrated by Yamamoto *et al*. (14) Furthermore, the expression of endothelin-1 in gingival epithelial cells is found to be enhanced during periodontal inflammation (14).

In nasal rhinitis, it has been suggested that endothelin-1 may establish a pro-inflammatory loop, which can become independent of the original stimulus and contribute to long-term inflammatory changes (15). Endothelin-1 may act on gingival tissues in a similar manner and this may explain the chronic inflammation of periodontitis.

Recently, has been found that the endothelin-1 level in gingival crevicular fluid increased with the progression of periodontal disease, and human gingival keratinocytes expressed mRNA for endothelins and their receptors (16).

Thus, in view of the aforementioned findings, this clinico-biochemical study was undertaken to estimate the gingival crevicular fluid endothelin-1 levels in subjects with clinically healthy periodontia, gingivitis and chronic periodontitis and, subsequently, after initial therapy (scaling and root planing) in the periodontitis subjects.

Material and methods

The study population consisted of 60 subjects (33 women and 27 men; age range: 27–39 years) attending the outpatient clinic of the Department of Periodontics, Government Dental College and Hospital, Bangalore, Karnataka, India. Written informed consent was obtained from those who agreed to participate voluntarily and ethical clearance was obtained from the institution's ethical committee. Patients with aggressive periodontitis, diabetes, hypertension, gastric mucosal or intraocular inflammatory conditions, sclerotic diseases, hepatic chirrhosis, coronary heart disease, chronic renal failure, gross oral pathology, habits of smoking, betel nut/areca nut chewing or alcoholism, and who had taken anti-inflammatory, antibiotic, H_2 blockers and/or immunosuppressive drugs, or who had received periodontal therapy in the preceding 6 mo, were excluded from the study.

Each subject underwent a full-mouth periodontal probing and charting, along with peri-apical 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 (17), pocket-probing depth, clinical attachment loss and radiograph evidence of bone loss, subjects were categorized into three groups. Group I (healthy) consisted of 20 subjects with clinically healthy periodontium, with a gingival index of 0, a pocket probing depth of ≤ 3 mm and clinical attachment loss of 0, with no evidence of bone loss on radiograph. Group II (gingivitis) consisted of 20 subjects who showed clinical signs of gingival inflammation, a gingival index of > 1, a pocket probing depth of ≤ 3 mm and had no attachment loss or radiographic bone loss. Group III (chronic periodontitis) consisted of 20 subjects who had signs of clinical inflammation, a gingival index of > 1, a pocket probing depth of ≥5 mm and attachment loss with radiographic evidence of bone loss. Patients with chronic periodontitis (group III) 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 wk after the treatment to constitute group IV (the after-treatment group). Descriptive statistics of the study groups in given in Table 1.

Site selection and fluid collection

All the clinical and radiological examinations, group allocation and sampling site selection were performed by one examiner (ARP) and the samples were collected on the subsequent day by a second examiner (CNG). This was undertaked to prevent the contamination of gingival crevicular fluid with blood associated with the probing of inflamed sites. Only one site per subject was selected as a sampling site in gingivitis and periodontitis groups (group II and group III), whereas, in the healthy group, to ensure the collection of an adequate amount of gingival crevicular fluid, multiple sites with absence of inflammation were sampled. In gingivitis patients, the site with the highest clinical signs of inflammation (i.e. redness, bleeding on probing and edema), in the absence of clinical attachment loss, was selected. In

Table 1. Descriptive statistics of the study groups

	Group I	Group II	Group III	Group IV
Age	32.20 ± 3.23 (year)	32.20 ± 3.17 (year)	32.80 ± 3.22 (year)	32.80 ± 3.22 (year)
GI	0.34 ± 0.09 (mm)	1.80 ± 0.39 (mm)	2.27 ± 0.37 (mm)	1.22 ± 0.20 (mm)
PPD	1.82 ± 0.31 (mm)	2.30 ± 0.46 (mm)	6.30 ± 0.0.41 (mm)	2.30 ± 0.32 (mm)
CAL	—	—	5.85 ± 1.08 (mm)	2.75 ± 0.63 (mm)
Absorbance	0.091 ± 0.005	0.089 ± 0.005	0.089 ± 0.006	0.094 ± 0.008

CAL, clinical attachment loss; GI, gingival index; PPD, pocket probing depth.

chronic periodontitis patients, sites with > 2 mm clinical attachment loss were identified using a Williams graduated periodontal probe, and the site showing the highest clinical attachment loss and signs of inflammation, along with radiographic confirmation of bone loss, was selected for sampling, and the same test site was selected for sampling after treatment.

On the subsequent day, after gently drying the area, supragingival plaque was removed without touching the marginal gingiva and the area was isolated using cotton rolls to avoid saliva contamination. Gingival crevicular fluid was collected by placing the microcapillary pipette at the entrance of the gingival sulcus, gently touching the gingival margin. From each group, a standardized volume of 1 μL was collected using the calibration on white color-coded 1–5-μL-calibrated volumetric microcapillary pipettes (Sigma–Aldrich, St. Louis, MO, USA). Each sample collection was allotted a maximum of 10 min and the sites which did not express any gingival crevicular fluid within the allotted time were excluded. This was carried out to ensure atraumatism, and the micropipettes that were suspected to be contaminated with blood and saliva were excluded from the study. The gingival crevicular fluid collected was immediately transferred to airtight plastic vials and stored at −70°C until required for assay.

Enzyme-linked immunosorbent assay

A commercial enzyme-linked immunosorbent assay (ELISA) kit (TiterZyme® EIA; Human Endothelin-1 Enzyme Immunometric Assay Kit, catalog no. 900-020; Assay Designs Inc., Ann Arbor, MI, USA) was used to detect the endothelin-1 concentrations in the gingival crevicular fluid samples. The sensitivity of the kit was 0.14 pg/mL. The gingival crevicular fluid samples were expelled from the microcapillary pipettes by a jet of air, using a blower provided with the micrpipettes, and further by flushing them using a fixed amount of diluent. After appropriate dilution, the samples and the standards [concentrations (pg/mL): 0, 0.78, 1.56, 3.1, 6.25, 12.5, 25, 50] provided with the kit were added to the wells of the multititer plate in duplicate. ELISA was performed, according to the manufacturer's instruction manual, at the Department of Biochemistry, Indian Institute of Sciences, Bangalore.

Results

The absorbance values obtained for the gingival crevicular fluid samples were below the absorbance value (0.098) obtained for the blank well (0 pg/mL). Thus, all the gingival crevicular fluid samples were negative for the presence of endothelin-1. The absorbance values obtained for the standards provided with the kit are given in Table 2.

Discussion

Endothelin-1, a 21-amino-acid vasoactive peptide acting as a pro-inflammatory cytokine, stimulates neutrophils to release elastase, activates mast cells and stimulates monocytes to produce a variety of cytokines such as interleukin-1α, interleukin-1β, interleukin-6, interleukin-8 and tumor necrosis factor-α, which play an important role in the initiation and progression of periodontal disease (13).

The study by Fujioka *et al.* (16) is the only study to quantify the endothelin-1 levels in gingival crevicular fluid in periodontal health and disease. However, the patient groups included only healthy controls and periodontitis patients. Also, the levels of endothelin-1 after periodontal therapy were not explored in that study. Hence, the present study was designed to quantify the endothelin-1 concentration in patients who were periodontally healthy, had gingivitis or periodontitis, and after nonsurgical periodontal therapy, to evaluate the effect of periodontal therapy on endothelin-1 levels.

Table 2. Absorbance values and corresponding endothelin-1 (ET-1) concentration observed in the assay procedure

Absorbance values	ET-1 concentration of the standards (pg/mL)
0.098	0
0.13	0.78
0.142	1.56
0.182	3.1
0.262	6.25
0.406	12.5
0.671	25
1.063	50

In the present study, the influence of age and gender of the subjects on the endothelin-1 levels was minimized by selecting subjects within the narrow age range of 27–39 years and by including an equal number of male and female subjects in all the groups. Furthermore, this study comprised four groups (healthy, gingivitis, chronic periodontitis and chronic periodontitis after treatment) compared with the previous study, where only two groups (healthy controls and periodontitis subjects) were included.

An extracrevicular (unstimulated) method of gingival crevicular fluid collection using microcapillary pipettes was employed in this study to avoid nonspecific attachment of the analyte, as seen with the filter paper technique used in previous studies. Furthermore, during removal of the gingival crevicular fluid, the micropipettes were flushed using a fixed amount of diluent to avoid attachment of the analyte to the walls of the microcapillary tube.

A sensitive ELISA method was employed to quantify endothelin-1 from selected sites to allow us to detect a small amount of endothelin-1 in the samples. Furthermore, in the present study we employed a more sensitive ELISA kit (sensitivity: 0.14 pg/mL) than that used in the previous study (sensitivity: 1 pg/mL).

We found that the gingival crevicular fluid samples in all the groups tested negative for the endothelin-1 molecule. These results were not in accordance with those of a previous study by Fujioka *et al.* (16), who reported increased levels of endothelin-1 in the gingival crevicular fluid of chronic periodontitis subjects (mean concentration: 388.6 pg/mL) when compared with healthy controls (mean concentration: 46.8 pg/mL) in the Japanese population. Fujioka *et al.* (16) found

endothelin-1 mRNA expression in cultured human gingival keratinocytes, human gingival fibroblast and human periodontal ligament cells, but failed to detect endothelin-1 peptide in conditioned medium of human gingival fibroblasts. They also found that the pro-inflammatory cytokines, interleukin-1β and tumor necrosis factor-α, did not induce endothelin-1 secretion from human gingival fibroblasts.

Yohn *et al*. (18) demonstrated that cultured human keratinocytes could synthesize endothelin-1 at the rate of ≈ 22 pg/1×10^6 cells spontaneously at 24 h. However, Bull *et al*. (19) failed to detect the production of endothelin-1 by keratinocytes using an indirect immunofluorescence technique.

The inability to detect endothelin-1 in the gingival crevicular fluid samples tested in our population could have several explanations.

(i) Endothelin-1 may be degraded in the gingival crevice by both bacterial- and host-cell-derived proteases, which is in accordance with the study by Sessa *et al*. (20), who found that polymorphonuclear leukocytes contain and/or release neutral proteases, which can both rapidly produce and degrade endothelin-1.

(ii) Endothelin-1 produced by activated cells in the gingival crevice may bind to endothelin-1 receptor-positive cells and may not be present in a free form in the gingival crevicular fluid.

(iii) As the presence of endothelin-1 is a result of spillover from a local release by cells in the gingival crevice, its concentration is relatively low in gingival crevicular fluid, added to which it has a short plasma half life of less than 1.5 min.

(iv) Finally, compared with previous studies, the differences in results obtained may be attributed to heterogeneous responses because of differences in race of the study population, as seen in studies of other cytokine responses (21).

Failure to detect endothelin-1, although not denying its role in periodontal disease, surely precludes its use as a 'potential biomarker' of periodontal disease progression. However, controlled longitudinal, prospective studies, involving a larger population and more solid-phase assays, are needed to verify this possibility.

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