ISSN: 2581-5989
PubMed - National Library of Medicine - ID: 101738774

International Journal of Dental Science and Innovative Research (IJDSIR)

IJDSIR : Dental Publication Service

Available Online at: www.ijdsir.com

Volume – 5, Issue – 3, June - 2022, Page No. : 278 - 285

Cystatin c levels in gingival crevicular fluid (GCF) in health and disease.

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Citation of this Article: Dr. Ashish Yadav, Dr. Mohamed Faizuddin, Dr. Gauravpal Singh, Dr. Saroj Nehra, "Cystatin c levels in gingival crevicular fluid (GCF) in health and disease", IJDSIR- June - 2022, Vol. – 5, Issue - 3, P. No. 278 – 285.

Type of Publication: Original Research Article

Conflicts of Interest: Nil

Abstract

Cysteine proteinases are considered as possible pathogenic agents for periodontitis because of their collagen degradation property. The importance of cysteine proteinases in periodontal disease has been recognized in recent years. This study was conducted to evaluate the association between periodontal disease status and cystatin C levels in gingival crevicular fluid from healthy and diseased periodontium. 90 Subjects were divided into three groups of 30 subjects each as with Healthy periodontium (A), Gingivitis (B) and Periodontitis group (C). Samples of gingival crevicular fluid were obtained from subjects of all the three groups by placing micro-capillary pipettes extra-crevicular Ly. Cystatin C levels were estimated using a commercially available enzyme linked immunosorbent assay (ELISA) kit. Mean concentration (in ng/ml) of cystatin C in group A was 412.18, 419.7 in group B and 269.98 in group C. There was negative correlation between cystatin C levels and bleeding index. Also lower levels of cystatin C were associated with higher values of clinical attachment levels (CAL). The results of our study indicate that cystatin C levels in GCF are inversely associated with periodontal disease and may be a potential indicator of periodontal disease severity. Our results suggest that measuring the levels of cystatin C in gingival crevicular fluid (GCF) may provide a useful tool in monitoring periodontal disease status.

Keywords: Cysteine proteinases, Cystatins, Gingival crevicular fluid, Periodontal diseases

Introduction

Periodontal disease is an infectious disease of supporting tissues of teeth caused by complex interaction between multiple bacterial species accumulating on tooth surface and host immune system. It is characterized by inflammation, tissue destruction and attachment loss. There is enough scientific data to suggest that tissue destruction is brought about by an array of proteolytic enzymes produced by pathogenic bacteria and cells of host tissues[1].

Proteinases are involved in the defense against microbes however, in this attempt these proteinases also contribute to periodontal tissue destruction by degrading extracellular matrix components. The degradation of extracellular matrix during periodontal disease is a multistep process that involves several proteolytic enzymes like collagenase, elastase and cysteine proteinases[2, 3, 4].

The importance of cysteine proteinases in periodontal disease has been recognized in recent years[5]. Cysteine proteinases are considered as possible pathogenic agents for periodontitis because of their collagen degradation property. Higher levels of cysteine proteinases in gingival crevicular fluid have been reported to be associated with increase periodontal destruction[6]. The activity of these proteinases is regulated by physiological inhibitors called cystatins[7]. Cystatins are of major interest because they inhibit collagen degrading cysteine proteinases.

Under normal conditions, there is a balance between the amount/activity of proteolytic enzymes and enzyme inhibitors, thereby keeping the activity of the secreted enzymes under control[8]. Therefore, when using proteolytic enzymes as indicators of tissue destruction; it would be more meaningful to measure the amount of inhibitors also.

Role of cystatins in periodontal health and disease has been investigated. Cystatin C was measured in gingival tissue from patients in various stages of periodontitis. Its concentration was reported to be significantly decreased at sites with higher pocket depths[9] and thus was proposed as a diagnostic indicator for the activity of periodontitis.

Barron et al have correlated the levels of salivary cystatins to their cysteine proteinase inhibitory activity in the whole saliva of periodontally diseased patients[10, 11]. Presence of cystatin A, cystatin C and kinogens has been demonstrated in the gingival tissues. There are only few published reports and studies till date[9, 12] which have estimated the levels of cystatins in gingival crevicular fluid (GCF).

Hence the present investigation is a further attempt to evaluate the association between periodontal disease status and cystatin C levels in gingival crevicular fluid as collection protocols of GCF are straightforward and non-invasive and can be performed at specific sites of interest in the periodontium[13]

Materials and methods

Study Population

The study population consisted of total 90 subjects belonging to both sexes and all the subjects selected were from outpatient clinics of department of periodontology, Mahatma Gandhi Dental College and Hospital, Jaipur, Rajasthan, India. There were 51 males and 39 females in the age range from 18 to 48 years. Subjects were divided into three groups of 30 subjects each as with Healthy periodontium(A), Gingivitis(B)and Periodontitis(C).

Exclusion criteria

- History of periodontal treatment received in the past six months.
- Under antibiotic treatment during the previous 6 months.

- History of underlying systemic disease
- Habit of smoking and tobacco chewing
- Pregnant woman and women on contraceptives.

Approval from the ethical committee of the institution was obtained, the nature and purpose of the study was explained to the subjects and written consent was taken. Selection of test site, detailed case history, clinical examination and supragingival scaling were done one day before the collection of gingival crevicular fluid (GCF). Data was recorded in a standard proforma. Details of the subjects divided into 3 groups (A, B and C) were as follows:

Group A (Healthy): It comprised of 30 subjects with clinically healthy periodontium, good oral hygiene status, no bleeding on probing and no loss of clinical attachment. Age ranged from 21 to 36 years. Total number of sites selected was 30 with one site in each subject.

Group B (Gingivitis): It comprised of 30 subjects with clinical signs of gingival inflammation, bleeding on probing present and without any clinical attachment loss. Age ranged from 18 to 38 years. Total number of sites selected was 30 with one site in each subject.

Group C (Periodontitis): It comprised of 30 subjects with presence of debris and calculus, clinical signs of gingival inflammation, bleeding on probing present, periodontal pocket depth of >3mm, and clinical attachment loss of >3mm. Age ranged from 28 to 48 years. Total number of sites selected was 30 with one site in each subject. Total sample size involved 90 sites with 30 each in group A, B and C.

Method of collection of gingival crevicular fluid (GCF)

The subjects were asked to gargle the mouth with water to cleanse the teeth of loosely adherent debris. Samples of GCF were obtained from subjects of all the three groups by placing colour coded calibrated, volumetric, micro capillary pipettes with 0-5µl range extra-crevicular Ly, obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

The test site selected was dried and isolated with cotton rolls.

The volumetric micropipettes were placed extra-crevicular Ly at the entrance of the gingival crevice, and for each subject standardized volume of 5µl was collected and measured within the micropipette. The pipettes which were contaminated with blood/saliva were discarded and the pipette with collected fluid was wrapped in a sterile aluminium foil to prevent oxidation, placed in a plastic vial and diluted with 45µl of normal saline, then immediately transferred to store at -70^0c, until analysed for cystatin C levels. Cystatin C levels were estimated using commercially available enzyme immunoassay (ELISA) kit. (Bio vendor laboratory medicine, Inc.)

Results

Study population consists of total 90 subjects belonging to both sexes. Age and sex distribution of the subjects is depicted in Table 1. There were 51 males and 39 females in the age range from 18 to 48 years. Subjects were divided into three groups of 30 subjects each as with Healthy periodontium (A), Gingivitis (B) and Periodontitis (C) group. There were 18 males and 12 females with the mean age of 27.10±4.12 years in group A, 18 males and 12 females with mean age of 28.50±7.12 years in group B and 15 males and 15 females with mean age of 35.80±6.60 years in group C.

Mean levels of cystatin C of each group is depicted in Table II. Concentration (in ng/ml) of cystatin C in group A varied between 141.84-1980.97 and mean was 412.18 (SE-76.57 and CI-95%). In group B the range was 148.85-1714.11 and mean concentration was 419.78 (SE-39.78 and CI-95%).

In group C the concentration range was 142.54-1059.52

and the mean was 269.98 (SE-32.69 and CI-95%). These values shows that cystatin C was significantly decreased in Group C in comparison to group A and group B (P=0.034) but no significant difference was observed between group A and B.

Coefficients for the correlation between levels of cystatin C and periodontal parameters is shown in table III. There is negative correlation between cystatin C levels and the clinical attachment level. Lower levels of cystatin C were associated with higher values of clinical attachment level (CAL). Correlation is statistically significant at $p<0.001$.

There is negative correlation between cystatin C levels and bleeding index. Higher values of bleeding index were associated with lower levels of cystatin C in group B and C but the correlation was statistically significant only in group C ($P<0.001$)

Regression analysis was conducted to evaluate linear and non-linear relationship of cystatin C with clinical attachment level. The R and P values for the regression are shown in Table IV. Both the linear and non-linear relationship is significant.

Discussion

Cysteine proteinases (CPs) comprise a family of proteolytic enzymes which are considered to play an essential role during periodontal breakdown. These enzymes are involved in intracellular protein degradation[14], pro-enzyme and pro-hormone processing[15], pathogenicity of malignant cells[16], breakdown of collagen[17] and bone resorption[18]. Cysteine proteinases are considered as possible pathogenic agents for periodontitis because of their activity against oral tissues[19]. This activity of cysteine proteinases is regulated by endogenous inhibitors called cystatins[20].

Cystatins are a group of proteins comprising a superfamily sub-divided into four families: Family-1, Family-2 Family-3 and Family-4 [21]. Cystatin C, a single chain protein of 120 amino acid residues, is a member of family-2 cystatins and a potent inhibitor of cysteine proteinases cathepsin B, H, L and S[5]. It is synthesized in most tissues of the body and has been found in all major biological fluids[22] and known to play an important role in regulating the activities of these proteinases[7.] Previous investigations have estimated cystatin C levels either in saliva or in diseased gingival tissue and was proposed as a diagnostic indicator for the activity of periodontitis. Saliva is a mixture of oral fluids and includes secretions from major and minor salivary glands, in addition to several constituents of non-salivary origin, such as serum and blood derivatives, GCF, food debris[23] and it could not be true representative of diseased condition in gingival crevice.

Harvesting the tissues from the diseased periodontal sites for the purpose of diagnosis is not feasible in routine clinical practice; hence the present investigation was designed to estimate the cystatin C levels in GCF. Gingival crevicular fluid (GCF) is a complex mixture of substances derived from serum, leukocytes, and structural cells of the periodontium and oral bacteria[24]. GCF is a unique window for analysis of periodontal condition. The periodontal disease activity is highest in the gingival tissue and GCF reflects the condition of the diseased gingival tissue.

The collection of GCF is a minimally invasive procedure and the analysis of specific constituents in the GCF provides a quantitative biochemical indicator for the evaluation of the local cellular metabolism that may reflect a person's periodontal health status[25]. In the present investigation, we have estimated the levels of cystatin C a potent inhibitor of cysteine proteinases in gingival crevicular fluid (GCF) from healthy and diseased periodontium to assess the relationship between cystatin C levels and severity of periodontal disease.

The results of our study showed that mean concentration (in ng/ml) of cystatin C in group

A was 412.18±76.57, in group B it was 419.78±39.78 and 269.98±32.69 in group C. These values show that among three groups concentration of cystatin C is significantly lower in Group C as compared to group A and group B indicating that there is an inverse relationship between concentration of cystatin C in GCF and periodontal destruction. This indicates that cystatin C levels in GCF represents severity of periodontal disease. The results of our study are comparable with results of Skal eric et al[9] who studied cystatin C concentration in gingival tissue obtained from diseased periodontium of different degree of inflammation as indicated by gingival index and probing depth. Their results showed that the total concentration of cystatin C is decreased in gingival samples taken from the sites with increased inflammation and probing depth. However the results of our investigation do not conform with those of Blankenvoorde et al[26] who have analysed different cystatin isoforms in gingival crevicular fluid and showed that cystatin C is not detected in any one of the GCF sample. The gross difference between our results and results of Blankenvoorde et al may be because of methodology used to estimate cystatin C concentration in GCF. In their study the method used was Immunoblot staining whereas we have utilized ELISA for estimation of cystatin C concentration in GCF. ELISA is a highly sensitive method which can detect micro and nano concentrations of the required constituents.

In one of the study by Sharma et al[27] had observed serum along with GCF cystatin C in periodontally diseased, healthy and gingivitis patients. Results stated that the mean concentration of cystatin C in serum as well as GCF was higher in patients with periodontal disease when compared with healthy periodontium which was not in accordance with our study results. Recent study by Christopher et al [28] showed that GCF cystatin C levels were more and highly significant in periodontitis group as compared to healthy group ($p<0.001$) This elevated levels of cystatin C in periodontitis may be due to cathepsin release stimulated by proinflammatory cytokines, thereby causing increased concentration of cystatin C to offset the surge in osteoclastic function and to prevent further periodontal degeneration. This was contrary to our finding. As mentioned earlier most of the studies have estimated the levels of cystatins in saliva in periodontal disease and though their results cannot be directly compared with our results, they indirectly support our observations. Baron et al who have estimated concentration of salivary cystatins have reported that cystatin levels were highest in periodontally healthy group as compared to diseased group[10]. Deshpande et al have also observed that the concentration of salivary cystatin C is less in periodontitis patients compared to healthy patients. These results are indirectly in accordance with results of our investigation[1]. Also Aditi shri Krishna dhage et al[29] had stated that salivary concentration of cystatin C was higher in healthy periodontium than in periodontitis patients. Abrahamson et al[30] who have studied cathepsin B activity in GCF has opined that increase in cathepsin activity in GCF from periodontally diseased sites may be because of degradation and depletion of cystatin C in highly proteolytic environment of diseased periodontium. GCF composition reflects the nature and amplitude of the host response to the microbial plaque challenge. Periodontal disease progression is highly dependent upon the host response, hence determination of GCF constituents level represents a practical approach for diagnostic utility[31].

Periodontal disease is episodic in nature with periods of

exacerbation and remission. Hence it is conceivable that some markers or group of markers might be associated with early acute inflammatory phase, while other markers might be more associated with chronic inflammatory stages, during which tissue breakdown occurs. The results of our study clearly indicate that cystatin C levels in GCF are inversely associated with periodontal disease and may be an indicator of periodontal disease. However in our study diseased activity at the site during harvesting of GCF and various isoforms of cystatins were not taken into consideration. It can be concluded that use of cystatin C levels in gingival crevicular fluid can further be explored for its diagnostic potential.

Conclusion

The results of our study indicate that cystatin C levels in GCF are inversely associated with periodontal disease and may be a potential indicator of periodontal disease severity. Also our results suggest that measuring the levels of cystatin C in gingival crevicular fluid (GCF) may provide a useful tool in monitoring periodontal disease status.

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Legend Tables

Table 1: Age and sex distribution of the subjects

Age and sex	Group A	Group B	Group C
Number of subjects	30	30	30
Age in years (Mean ± SD)	27.10±4.12	28.50±7.12	35.80±6.60
Sex	Male=60.0% Female=40.0%	Male=60.0% Female=40.0%	Male=50.0% Female=50.0%

Table 2: Comparison of levels of cystatin C between different groups.

Cystatin C	Group A	Group B	Group C
Range	141.84-1980.97	148.85-1714.11	142.54-1059.52
Mean ± SE	412.18±76.57	419.78±39.78	269.98±32.69
95% CI	255.57-568.78	297.50-542.06	203.17-336.86
Significance	Cystatin C is significantly reduced in Group C with P=0.034		

Table 3: Correlation of Cystatin C with Bleeding Index and CAL in Group C Correlation obtained by Spearman correlation

Pair	Spearman Correlation	p value
Cystatin C vs Bleeding Index in Group B	-0.292	0.117
Cystatin C vs Bleeding Index in Group C	-0.955	<0.001
Cystatin C vs CAL in Group C	-0.991	<0.001

Table 4: Lenear and Non-linear relationship of Cystatin C with CAL

Regression analysis	Linear	Non-linear
Function	543.76-68.90xCAL	$732.09xCAL^{-0.871}$
R^2	0.416	0.876
P value	<0.001	<0.001