The Evaluation of Cystatin C, IL-1β, and TNF-α Levels in Total Saliva and Gingival Crevicular Fluid From 11- to 16-Year-Old Children

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Background: The aim of this study was to evaluate the levels of cystatin C, interleukin-1β (IL-1β), and tumor necrosis factor-alpha (TNF-α) in the total saliva and gingival crevicular fluid (GCF) of periodontally healthy children (PHC) and children with gingivitis (CG) who were between 11 and 16 years old.

Methods: The study was carried out with 10 PHC and 25 CG. Unstimulated total saliva and GCF samples were obtained. Clinical parameters, including probing depth (PD), clinical attachment loss (CAL), plaque index (PI), gingival index (GI), and gingival bleeding index (GBI), were assessed. GCF samples were collected from four maxillary upper incisors. After sampling, biochemical analyses were performed using latex particle–enhanced turbidimetric immunoassay for cystatin C and enzyme-linked immunosorbent assay for IL-1β and TNF-α. The multivariate analysis of variance test was used for statistical evaluation.

Results: In total saliva, cystatin C and TNF-α levels were higher in PHC, and IL-1β levels were higher in CG, but the differences were not statistically significant. In GCF, cystatin C levels were higher in PHC ($P > 0.05$), whereas TNF-α and IL-1β levels were higher in CG ($P > 0.05$). In the CG group, there were positive correlations between the GCF cystatin C level and the PI of the sampled site ($r = 0.488$; $P < 0.05$); also, GCF IL-1β ($r = 0.603$; $P < 0.05$) and TNF-α ($r = 0.456$; $P < 0.05$) levels were positively correlated with PD and CAL. For the whole mouth and the sampled sites, PI, GI, GBI, PD, and CAL values were higher in CG ($P < 0.05$), but no significant differences were detected between GCF volumes of the two groups.

Conclusions: To the best of our knowledge, this study represents the first evaluation of cystatin C in the gingival disease mechanism in children. Our results showed that total saliva and GCF cystatin C levels were higher in PHC ($P > 0.05$), but there was no correlation between cystatin C levels and IL-1β or TNF-α levels in total saliva or GCF. *J Periodontol 2008;79:854-860.*

KEY WORDS

Children; cystatin C; gingival crevicular fluid; interleukin-1 beta; saliva; tumor necrosis factor-alpha.

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The prevalence of gingivitis peaks at ~11 years of age and then decreases slightly with age over the following 4 years. The onset of puberty and the increase in circulating levels of sex hormones have been offered as an explanation for the increase in gingivitis seen at this time.[1] Gingivitis in children is often characterized by gingival inflammation without the detectable loss of bone or clinical attachment.[2] Components of microbial dental plaque can activate the local host response by inducing the infiltration of inflammatory cells, including lymphocytes, macrophages, and polymorphonuclear leukocytes.[3] In the gingival crevice, lipopolysaccharide triggers monocytes to release inflammatory mediators that increase the local destruction of the connective tissue structure. Accordingly, levels of monocyte inflammatory mediators, including prostaglandin E_2, interleukin (IL)-1, and tumor necrosis factor (TNF), in gingival crevicular fluid (GCF) may represent ideal markers of disease activity.[4]

IL-1 is a proinflammatory cytokine with a large array of biologic activities. There are two principal forms of IL-1 that have agonist

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activity, IL-1α and IL-1β, whereas IL-1 receptor antagonist functions as a competitive inhibitor.[5] IL-1β, the predominant form of IL-1 in periodontal tissues, is produced mainly by macrophages.[6] TNF is another cytokine that has been studied extensively in adults. The term refers to two associated proteins, TNF-α and lymphotoxin-α, also known as TNF-β.[5] TNF-α is produced mainly by macrophages in response to antigens such as lipopolysaccharides.[6]

Cysteine proteinases can degrade many extracellular matrix molecules, including some collagenous components; although normally intralysosomal, they may also be secreted.[7] Many normal and pathologic processes are controlled by the balance between proteinases and their inhibitors. The activities of cysteine proteinases are controlled by naturally occurring inhibitory proteins, such as α_2-macroglobulin and cystatins, which protect host tissues from destructive proteolysis by cysteine proteinases of host, bacterial, and viral origin.[8] Cystatins bind tightly and reversibly to cysteine proteinases. The most investigated of these is cystatin C, which potently inhibits papain and lysosomal cysteine proteinases and has been found in all tested human biologic fluids. By contrast, cystatins S, SA, SN, and D are only found in whole saliva, glandular saliva, and tear fluid.[9]

Lupi et al.[10] found cystatin SN, S, S1, and S2, but not cystatin C, in the total saliva of healthy subjects. Henskens et al.[11] similarly could not detect cystatin C in GCF. In another study,[12] cystatin C was detected in saliva samples but not in GCF samples.

Additionally, we note that elevated concentrations of inflammatory mediators in the periodontal environment may be responsible, at least in part, for the conversion of established gingivitis lesions into more advanced states involving connective tissue and attachment loss.[13] Thus, in addition to correlations among cystatin C, IL-1β, and TNF-α, the present study evaluated correlations between clinical parameters and levels of these markers in the total saliva and GCF of children between 11 and 16 years of age.

MATERIALS AND METHODS

Subject Selection and Clinical Procedures

Between September 2005 and October 2006, 10 periodontally healthy children (PHC) (four females and six males; mean age: 13.6 years) and 25 children with gingivitis (CG) (18 females and seven males; mean age: 13.56 years) were selected from patients newly referred to the Pediatric Dentistry Department, Faculty of Dentistry, University of Gazi. Informed consent was obtained from the children's parents, and total saliva and GCF sampling and clinical procedures were explained fully before the study. The protocol was approved by the Ethics Committee of the Faculty of Dentistry, University of Ankara. Periodontal disease status was determined by clinical examination and radiographs. All children were in good general health, and none had received periodontal therapy or medication during the past 6 months; no participants had a history of systemic disease. They were not on any medication that could affect the manifestations of periodontal disease, such as chronic antibiotics, phenytoin, cyclosporin, anti-inflammatory drugs, systemic corticosteroids, or calcium channel blockers.

Collection of Total Saliva and GCF

Prior to the clinical measurements, unstimulated total saliva was collected between 10:00 am and 12:00 pm by spitting. Subjects refrained from eating, drinking, and oral hygiene for 2 hours prior to saliva collection. Saliva was immediately cleared by centrifugation (11,900 × g for 10 minutes) at room temperature. The supernatant was frozen at –20°C until analyzed.

The plaque index (PI)[14] was recorded after the collection of total saliva. In the CG group, GCF samples were collected from four maxillary upper incisors that were affected by gingivitis. The area was isolated by cotton rolls, and the teeth and marginal gingiva were dried with air before sampling.[15] Upper teeth were chosen to avoid contamination by saliva during GCF sampling; if a lateral tooth was missing, samples were taken from a canine tooth. After recording of the PI and removal of supragingival plaque were completed, paper strips‖ were inserted for 30 seconds into the buccal crevice to a level of 1 mm below the gingival margin.[16] Care was taken to avoid mechanical injury to the gingival tissues. Strips contaminated by bleeding or exudates were discarded.[17,18]

The paper strips were placed into coded, sealed plastic microcentrifuge tubes and then stored at –20°C until analyzed. Paper strips from different sites in each patient were pooled.

Periodontal Examination

A periodontal examination was performed by a pediatric dentist at the beginning of the dental visit. Assessment of clinical parameters included probing depth (PD), clinical attachment loss (CAL), PI, gingival index[19] (GI), and gingival bleeding index[20] (GBI). To avoid the contamination of filter paper strips with blood, GI, GBI, PD, and CAL were measured after GCF collection. All periodontal disease measurements were performed in four quadrants. PD and CAL levels were measured in eight teeth, including #8, #9, #24, #25, #3, #14, #19, and #30 with a periodontal probe¶ calibrated in millimeters, whereas other parameters, including PI, GI, and GBI, were measured in the entire mouth. PD and CAL were measured at six sites per tooth (mesial-median-distal

‖ Periopaper, ProFlow, Amityville, NY.
¶ Prestige, Sialkot, Pakistan.

buccal; mesial-median-distal palatal or lingual), and PI, GI, and GBI were measured at four sites per tooth (mesial, distal, buccal, and palatal sites on upper teeth; mesial, distal, buccal, and lingual sites on lower teeth).

Biochemical Analyses

Filter paper strips were placed in 50 μl phosphate buffered saline (pH 7.4) and incubated for 1 hour at 4°C. The fluid from the paper strip was recovered by centrifugation (11,900 × g for 10 minutes) and stored frozen at –20°C until used. Frozen saliva samples were mixed thoroughly after thawing and recentrifuged before analysis. Repeated freeze-thaw cycles were avoided.

Cystatin C Assay

Total saliva and GCF levels of cystatin C were determined by the latex particle–enhanced turbidimetric method using a cystatin C kit[#] on a biochemical autoanalyser instrument[**] according to the manufacturer's instructions. The absorbance was measured at 552 nm, and the cystatin C concentration of each sample was calculated from the calibration curve.

TNF-α and IL-1β Assay

Total saliva and GCF levels of TNF-α and IL-1β were determined by using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kit[††] as described.[21] The absorbance values were determined by using an ELISA reader[‡‡] at 450 nm. A standard curve was constructed by using standards provided in the kits, and the cytokine concentrations were calculated from the standard curve.

Statistical Analyses

Data were entered into a personal computer and analyzed using a software program.[§§] A multivariate analysis of variance test was used to analyze the differences between PHC and CG. The correlations among the mediator levels and clinical parameters were analyzed using Pearson's correlation analysis. A P value <0.05 was considered statistically significant.

RESULTS

Total Saliva and GCF Measurements

TNF-α was undetectable in the total saliva of one PHC and four CG, as was IL-1β in two PHC and three CG. Statistical comparisons of cystatin C, TNF-α, and IL-1β levels in the total saliva and GCF from PHC and CG are shown in Tables 1 and 2, respectively.

Although there was not a statistically significant difference, total saliva cystatin C and TNF-α levels were higher in PHC, whereas IL-1β levels were higher in CG (P >0.05) (Table 1).

GCF cystatin C levels were higher in PHC, whereas TNF-α and IL-1β levels were higher in CG, but again the differences were not statistically significant (Table 2).

Clinical Parameters and Correlation Analyses

Statistically significant differences were observed between whole-mouth and sampled-site PI, GI, GBI, PD, and CAL values in the two groups. As expected, these values were higher in CG (Tables 3 and 4) (P <0.05). GI and GBI of the sampled sites in CG were greater than zero, implying that all of the sampled sites were affected by gingivitis. No significant difference was detected in GCF volumes between the two groups (Table 2).

Correlations between biochemical markers and clinical parameters were seen in the CG group, but not in the PHC group. The correlation between the GCF cystatin C level and the PI of sampled sites was positive, and the difference was statistically significant (r = 0.488; P <0.05) (Fig. 1). GCF IL-1β (Fig. 2) and TNF-α (Fig. 3) levels were positively correlated with PD and CAL, with statistically significant differences (r = 0.603; P <0.05 and r = 0.456; P <0.05, respectively).

DISCUSSION

This study evaluated two host-response cytokines, IL-1β and TNF-α, and the protease inhibitor cystatin C at individual sites of periodontal health and gingivitis. Previous studies[5-7] examining periodontal mediators have generally focused on adult populations and compared levels in tissues or fluids of periodontally healthy subjects with those in subjects with periodontitis. Consequently, data on levels of periodontal disease markers in children with gingivitis remain inadequate. To our knowledge, there is no information about the levels of cystatins in the total saliva or GCF of children.

Our study showed that total saliva cystatin C levels were higher in PHC, although the difference was not statistically significant. This is contrary to the finding of Henskens et al.,[22] who reported that high cystatin concentrations in saliva were related to the presence of gingivitis and periodontitis. Other articles from this group[11,23] have supported the same conclusion.

However, in accord with our findings, Aguirre et al.[24] reported that statistical analysis showed no significant difference in the levels and activity of salivary cystatins in periodontally healthy and diseased individuals. Baron et al.[25] found total cystatin inhibitory activity and total salivary cystatin concentration to be lower in the periodontally diseased patients than in the controls.

One study[26] suggested that total cystatin activity and cystatin C concentrations of total saliva samples

DAKO, Glostrup, Denmark.
** Cobas Integra 800, Roche, Mannheim, Germany.
†† Biosource, Ontario, CA.
‡‡ LP 400, Pasteur Diagnostic, Chaska, MN.
§§ SPSS 11.0, SPSS, Chicago, IL.

Table 1.

Statistical Comparison of Cystatin C, TNF-α, and IL-1β Levels in Saliva of PHC and CG

	PHC			CG			
	n	Mean	SD	n	Mean	SD	*P*
Cystatin C (mg/l)	7	3.721	2.1740	19	2.987	1.1392	0.407
TNF-α (pg/ml)	7	623.386	395.1006	19	321.163	335.0432	0.063
IL-1β (pg/ml)	7	2.270	1.5953	19	5.587	5.4410	0.130

Table 2.

Statistical Comparison of Cystatin C, TNF-α, and IL-1β Levels in GCF of PHC and CG

	PHC			CG			
	n	Mean	SD	n	Mean	SD	*P*
Cystatin C (mg/l)	10	1.1450	0.0611	25	1.1008	0.0869	0.153
TNF-α (pg/ml)	10	27.690	10.8294	25	32.072	9.6118	0.248
IL-1β (pg/ml)	10	14.0000	9.9482	25	17.8732	10.0523	0.309
GCF volume (μl)	10	0.23	0.15	25	0.20	0.13	0.647

Table 3.

Statistical Comparison of Whole-Mouth Clinical Parameters in PHC and CG

	PHC			CG			
	n	Mean	SD	n	Mean	SD	*P*
PI	10	0.1883	0.2346	25	0.5563	0.5410	0.047*
GI	10	0.0000	0.0000	25	0.4832	0.4959	0.005*
GBI	10	0.0000	0.0000	25	0.1635	0.1904	0.011*
PD	10	1.0728	0.0689	25	1.3029	0.3142	0.030*
CAL	10	1.0728	0.0689	25	1.3029	0.3142	0.030*

* $P < 0.05$.

Table 4.

Statistical Comparison of Clinical Parameters of Sampled Sites (four maxillary incisors) in PHC and CG

	PHC			CG			
	n	Mean	SD	n	Mean	SD	*P*
PI	10	0.0375	0.0790	25	0.4529	0.6006	0.038*
GI	10	0.0000	0.0000	25	0.6125	0.5257	0.001*
GBI	10	0.0000	0.0000	25	0.2550	0.2134	0.001*

* $P < 0.05$.

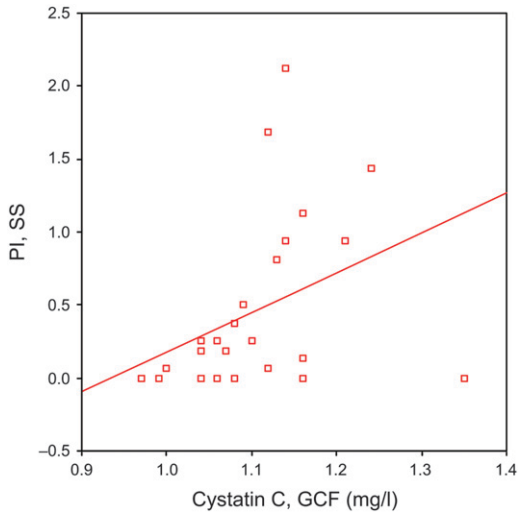

Figure 1.
Correlation between GCF cystatin C level and PI of sampled sites (SS) in CG (r = 0.488; P <0.05).

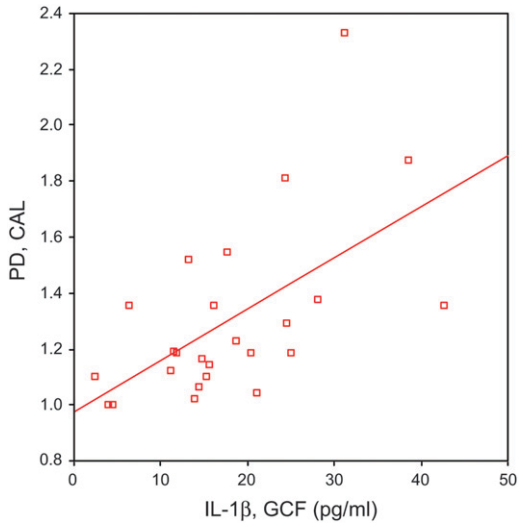

Figure 2.
Correlation between GCF IL-1β level and PD and CAL in CG (r = 0.603; P <0.05).

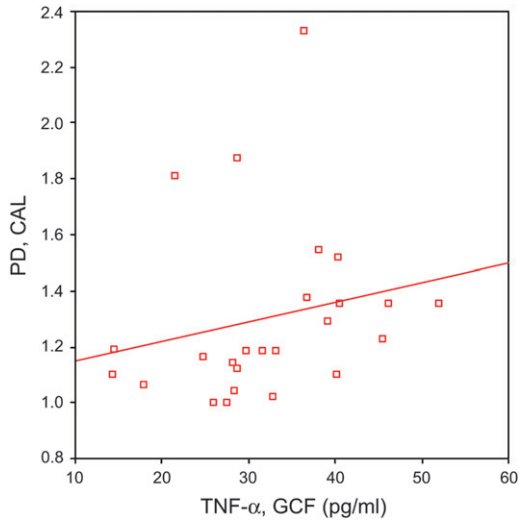

Figure 3.
Correlation between GCF TNF-α level and PD and CAL in CG (r = 0.456; P <0.05).

collected after periodontal treatment decreased to values characteristic of normal healthy controls. To the contrary, Lie et al.[27] found that at the end of a 14-day experimental gingivitis period, smokers showed a decrease in cystatin activity and cystatin C levels. Similarly, van Gils et al.[28] reported that salivary cystatin C concentrations were reduced after a 21-day experimental gingivitis period, although the difference was not statistically significant.

To support our findings, we also evaluated the levels of cystatin C in GCF of children; they were non-significantly higher in PHC. We suggest that lower cystatin C levels in CG may lead to higher levels of cysteine proteinase activity and contribute to periodontal disease. This is in accord with the findings of Skaleric et al.[29] and the observation of Lah et al.[30] that cystatin C concentrations are significantly lower at sites with greater probing depths.

In the literature, levels of TNF-α and IL-1β have generally been analyzed in GCF of adults. In the current study, these two cytokines were analyzed in the total saliva and GCF of children. Relatively few studies[31-34] have examined IL-1β and TNF-α in this age group.

We found that total saliva TNF-α levels were higher in PHC, whereas GCF TNF-α levels were higher in CG ($P>0.05$). IL-1β levels showed a different pattern, being higher in the total saliva and GCF of the CG group. No differences were statistically significant, however.

It was reported that there is a highly significant correlation between levels of IL-1β and TNF-α in tissue samples and cultured blood mononuclear cells from periodontal patients.[21,32,35] However, we could not find any correlation between these two markers in total saliva or GCF.

Total saliva is a complex mixture derived from the major and minor salivary glands, along with contributions from the GCF, oral bacteria, cells, and other sources.[4,36] In line with the results of our study, Wozniak et al.[37] showed that TNF-α could be detected in total saliva samples of healthy adult subjects, and Rossomando et al.[38] suggested that TNF might be found in sites prior to clinically observable disease. The latter group went on to suggest that TNF might prove to be a suitable indicator for preclinical periodontal disease. If so, our healthy subjects who demonstrated higher TNF-α levels in their total saliva may

be prone to periodontal disease. Additionally, the higher amount of TNF-α in the total saliva of PHC in our study may reflect different cellular sources of TNF-α in the two groups.

Yakovlev et al.[33] analyzed IL-1β and TNF-α in gingival biopsies of prepubertal children (6 to 14 years of age), young adults (18 to 35 years of age), and mature adults (36 to 54 years of age). Only in the young adult group were levels of IL-1β significantly higher in inflamed gingiva compared to non-inflamed gingiva, whereas there was no significant difference in TNF-α at any age. Sampling of gingival tissue rather than GCF may account for the differences from our results.

In line with our findings in children, higher GCF IL-1β levels were reported in adult patients with gingivitis than in periodontally healthy subjects.[39] Lerner et al.[31] reported increased GCF levels of IL-1 in teenaged children with periodontitis, but they did not measure levels in gingivitis.

Ullbro et al.[34] evaluated the levels of IL-1β and TNF-α in GCF of young patients with Papillon-Lefèvre syndrome. They were increased in these patients, but only the elevation of IL-1β was statistically significant.

For whole-mouth clinical parameters and those measured at specific sites (PI, GI, GBI, PD, and CAL), values were higher in CG, and the differences were statistically significant. No significant difference in GCF volume was detected between the two groups. In line with our findings, Kurtis et al.[18] reported that PI, GI, PD, and CAL values were lower in the control group than in subjects with periodontitis.

We found a positive correlation between the PI of sampled sites and the GCF cystatin C level ($r = 0.488$; $P < 0.05$). We expected the opposite result. However, reports[40-42] in the literature suggested that plaque scores and the effectiveness of oral hygiene do not correlate well with the severity of periodontal disease and clinical status. This leads us to agree with the suggestion that a low PI indicates good oral hygiene, but provides little information about inflammation.[43]

In the current study, IL-1β ($r = 0.603$; $P < 0.05$) and TNF-α ($r = 0.456$; $P < 0.05$) levels in GCF were positively correlated with PD and CAL. Mogi et al.[44] and Yavuzyilmaz et al.[17] reported a similar positive correlation between IL-1β levels in GCF and mean PD. IL-1β levels in saliva correlated positively with PD and CAL.[36]

CONCLUSIONS

Levels of cystatin C, IL-1β, and TNF-α in the total saliva and GCF of children have not been investigated. This study demonstrated that alterations in the levels of these three mediators can be observed in gingivitis and even in PHC. However, the study was limited to children with permanent dentition at an age when gingivitis is common. Further studies are needed to evaluate the levels of these markers in children of different age groups. Because early diagnosis ensures the greatest chance for successful treatment, it is important that children receive a periodontal examination as part of their routine dental visits.

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