Elastase and metalloproteinase-9 concentrations in saliva in patients with chronic periodontitis

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Abstract

Elastase and metalloproteinase-9 (MMP-9) are two of numerous proteolytic enzymes released by neutrophilic granulocytes in the course of periodontitis. The aim of the study was to determine the concentrations of elastase and MMP-9 in saliva in patients with chronic periodontitis compared to healthy individuals.

The enzyme-linked immunosorbent assay method was employed to determine the concentrations of elastase and MMP-9 in saliva in patients with chronic periodontitis and with pocket depth (PD) ≥ 6 mm and PD < 6 mm, as well as in saliva of healthy individuals. Significantly higher concentrations of elastase and MMP-9 were observed in patients with periodontitis compared to healthy individuals (p < 0.01). Also a significant difference in elastase concentration in saliva was observed between the PD ≥ 4 mm and PD < 6 mm groups and between the PD ≥ 6 mm and control groups, and statistically significant differences in MMP-9 concentrations between the PD ≥ 6 mm and control groups. No statistically significant differences were observed between the PD < 6 mm and control groups for elastase concentrations in saliva as well as between the PD ≥ 6 mm and PD < 6 mm groups, and also between the PD < 6 mm and control groups for MMP-9 concentrations in saliva. Elastase and MMP-9 concentrations in saliva can be considered as biochemical indicators of severity of periodontitis.

Key words: *chronic periodontitis, elastase, metalloproteinase-9.*

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Introduction

The traditional diagnosis of periodontal disease is based on clinical and radiological examination. Ideal diagnosis should be based not only on clinical parameters of periodontal tissues at the moment of examination, but also on clinical and biochemical indices which could indicate a potential progression of the disease. Unfortunately, until now no ideal indicators have been developed which could predict progression of the disease, primarily in patients with possible periodontal lesions at early age, or which could detect a risk of such lesions at the subclinical level. We still have at our disposal solely methods of examining the clinical attachment loss (CAL) and radiological methods of alveolar bone loss (BL) assessment. Unfortunately, observation of these indicators in time is possible only after a few millimetres of clinical attachment or bone have been lost. Therefore, for many years researchers have been trying to develop diagnostic tests which could help to identify patients (or certain sites in a patient's periodontium) particularly susceptible to occurrence and fast progress of periodontal disease [1].

Numerous studies were devoted to determining specific bacterial species (strains) present in dental plaque, which are responsible for faster progression of periodontal disease of its occurrence at an early age. Nowadays, the so-called red complex bacteria *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and green complex *Aggregatibacter actinomycetemcomitans* are regarded as indicator species [2-6].

Many clinical studies tried to establish various substances in gingival crevicular fluid, saliva or gingival tissue which could be indicators of disease progression. These substances include such inflammatory mediators as PGE2, interleukins e.g. IL-1β, enzymes e.g. elastase and metalloproteinases [7, 8]. These substances are secreted by periodontal cells (fibroblasts, keratinocytes) and inflammatory cells. In the light of contemporary studies it is known that although the primary etiological factor in chronic periodontitis is bacteria, most destructive processes within the bone and connective tissue occur after initiation of immuno-inflammatory processes within the above-mentioned tissues.

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Elastase is a neutral serine proteinase (endopeptidase) "stored" in azurophilic granules of granulocytes, at the number of 3 pg per cell. The enzyme is capable of degrading a large spectrum of various molecules in human tissues, including periodontal tissues, such as collagen, laminin, fibronectin, proteoglycans and elastin [9]. Elastase seems to play a particular role in the early stages of periodontal disease, as contrary to e.g. collagenases it can destroy intact non-collagenous proteins. In healthy periodontium these proteins surround collagen fibers protecting them against activity of collagenases [10, 11].

Elastase is one of numerous proteolytic enzymes (proteases) released by neutrophilic granulocytes as a result of such phenomena as tissue destruction or bacterial infection. Elastase is present in granules of granulocytes in the form of both a proenzyme and an active enzyme, however their activating mechanisms are not fully known. Outside the cell, elastase activity control is performed mainly by serine proteinase inhibitor – alpha-1-antitrypsin (A1AT), produced and released also by neutrophils [12].

Neutrophilic granulocytes are dominant leukocytes in epithelium of periodontal pockets and adjacent periodontal tissues. They protect periodontal tissues against bacterial infections and subsequent tissue destruction by means of oxidative and non-oxidative mechanisms. The latter seem to be of the highest significance in anaerobic conditions of the periodontal pocket. Most antibacterial substances are located in azurophilic granules of neutrophilic granulocytes. They are capable of destroying phagocytized bacteria, but they can be also released extracellularly during phagocytosis [13].

Numerous *in vitro* studies confirmed that neutrophilic granulocytes activated by bacteria from dental plaque – release lysosomal enzymes, including elastase [14-18]. Elastase reaches one of the highest levels among proteinases marked quantitatively in gingival crevicular fluid (GCF).

Matrix metalloproteinases are a group of proteolytic enzymes, acting extracellularly. Initially, it was believed that each metalloproteinase has its own substrate. Now it is known that they can decompose a variety of extracellular matrix proteins. Substrates for metalloproteinase 9 (MMP-9) are, inter alia, type IV, V, VII, X, XIV collagen, gelatin, elastin, fibronectin, osteonectin, plasminogen. Under physiological conditions, MMP-9, as well as other metalloproteinases, is produced by connective and epithelial tissue cells, and is involved in their remodeling and healing. In the course of periodontitis, cells exposed to products released by bacteria, cytokines and growth factors, secrete higher amounts of metalloproteinases [19, 20]. Metalloproteinase 9 is also secreted by inflammatory cells. Metalloproteinases secreted by immune cells such as PMN leukocytes and monocytes/macrophages allow them to migrate from the blood vessels and to penetrate tissues, which is the essence of the processes occurring in inflammatory diseases. Excessively produced and secreted metalloproteinases destroy components of connective tissue extracellular matrix. Moreover, inflammatory cells stimulated by bacteria secrete cytokines which in turn stimulate stationary cells to produce metalloproteinases. Studies have shown that PMN leukocytes stimulated in vitro secrete MMP-8 and MMP-9 from intracellular granules within a few seconds from commencement of stimulus action [21-23].

The gingival (periodontal) crevicular fluid, which is a transudate from serum or inflammatory transudate, is a reflection of blood serum ingredients as well as cellular response and processes occurring in marginal periodontium. Examination of gingival crevicular fluid plays an important role in scientific research on pathophysiology of periodontal disease. Cellular (e.g. granulocytes) and humoral (e.g. enzymes) elements of crevicular fluid penetrate into saliva. An analysis of GCF-originated mediators in mixed saliva could be a fast screening method for periodontal disease [24].

Aim

The aim of the study was to assess elastase and MMP-9 concentrations in saliva in patients with chronic periodontitis compared to individuals with healthy periodontium, and to determine the correlation between concentrations of the two enzymes in saliva.

Material and methods

The study included a group of 32 patients (20 females and 12 males) with diagnosed chronic periodontitis [25]. Inclusion criteria were ≥ 18 years of age, ≥ 10 erupted teeth, clinical attachment loss (CAL) ≥ 3 mm. Within the group two subgroups were distinguished according to periodontal pocket occurrence: pocket depth (PD) ≥ 6 mm and PD < 6 mm. Average age was 41.76 years. None of the patients reported any coexisting systemic diseases and was subject to long-term pharmacological treatment. Individuals who during the previous three months were treated with antibiotics or anti-inflammatory medications, especially non steroidal anti-inflammatory drugs and aspirin, were excluded from the study. Exclusion criteria were smoking, pregnancy, lactation, too. The control group consisted of 13 individuals (7 females and 6 males) with healthy periodontium – without clinical attachment loss, without periodontal pockets > 3 mm and with bleeding index < 10%. Due to the above criteria, average age in the control group was 32.2 years.

Five milliliters of whole mixed expectorated saliva was collected from each individual between 9.00 and 13.00 hours (at least 2 hours after a meal) according to a modification of the method described by Navazesh [26]. Patients rinsed their mouth with tap water and expectorated whole saliva into sterile tube while seated in an upright position. The collected material was frozen at –20°C and stored in that temperature until biochemical tests were performed. Samples were analyzed within 3 months of collection.

Elastase and MMP-9 concentrations in saliva was determined in duplicate for each subject with enzyme-linked immunosorbent assay kits ELISA (R&D Systems Minneapolis MN, USA), according to the manufacturer's directions. Standards were included on all runs and all results are reported within the linearity of the assays. The test sensitivity was 1.98 ng/ml and 0.156 ng/ml, respectively.

Statistical analysis

The concentrations of elastase and MMP-9 in three groups of patients (PD ≥ 4 mm, PD < 4 mm and control) was compared. In order to determine if the differences between the groups are significant (after checking for normality of the distribution with Kolmogorov-Smirnov test), one-way analysis of variance was used. In the case of a rejection of the hypothesis about lack of differences in the level of examined parameters between groups, Tukey's multiple comparison test was used to assess differences between group pairs. Interdependence between elastase and MMP-9 concentrations was assessed with Pearson correlation coefficients. All calculations were performed with SPSS 12.0 statistical software package.

Results

The mean concentration of elastase in saliva in patients with periodontitis was 11204.44 ng/ml, whereas in the control group the result was 2849.38 ng/ml. Metalloproteinase 9 concentration in saliva in patients with periodon-

titis was 418.97 ng/ml, in the control group the result was 221.15 ng/ml. The differences were statistically significant ($p < 0.01$ for elastase and $p = 0.025$ for MMP-9) (Table 1). After dividing the study group into two subgroups, PD ≥ 6 mm and PD < 6 mm, the distribution of examined parameters did not significantly differ from the normal one. Variance analysis showed significant differences between examined parameters (p for variance analysis < 0.001) (Table 2). In Tukey's multiple comparison test significant differences were observed in the elastase concentration in saliva between the PD ≥ 6 mm and PD < 6 mm groups as well as between PD ≥ 6 mm and control groups, however no statistically significant differences were observed between the PD < 6 mm and control groups (Table 3). The same test disclosed significant differences between the PD ≥ 6 mm and control groups for MMP-9. The PD ≥ 6 mm and groups as well as the PD < 6 mm and control groups did not indicate statistically significant differences (Table 3).

Moreover, a significant negative correlation was observed between age and the number of teeth in the PD ≥ 6 mm and PD < 6 mm groups, and a statistically significant positive correlation between elastase and MMP-9 concentrations in the PD ≥ 6 mm group. No significant correlations of the above mentioned parameters were observed in the control group (Table 4).

Discussion

Cross-sectional examinations showed an increased level of elastase in diseased sites compared to healthy sites

Table 1. Comparison of elastase and MMP-9 concentrations in saliva between the whole study and control groups

	Group	N	Mean	SD	p
Elastase	control	13	2849.38	2418.146	< 0.001
	study	32	11204.44	9365.738	
MMP-9	control	13	221.15	156.607	0.025
	study	32	418.97	414.582	

Table 2. Comparison of elastase and MMP-9 concentrations in saliva between PD ≥ 6 mm, PD < 6 mm and control groups

		N	Mean [ng/ml]	SD	Min [ng/ml]	Max [ng/ml]	p_{ANOVA}
Elastase	PD ≥ 6mm	16	17870.1	8804.2	5500	37 000	< 0.001*
	PD < 6mm	16	4538.8	2996.9	320	9700	
	control	13	2849.4	2418.1	330	6500	
MMP-9	PD ≥ 6mm	16	565.8	493.6	32	1710	0.017**
	PD < 6 mm	16	272.2	256.1	60	953	
	control	13	221.2	156.6	15	535	

In Tukey's multiple comparison test, significant differences were observed between PD ≥ 6 mm and PD < 6 mm groups as well as between PD ≥ 4 mm and control groups, no statistically significant differences were observed between PD < 6 mm and control groups

**In Tukey's multiple comparison test, significant differences were observed between PD ≥ 6 mm and control groups. The PD ≥ 6 mm and PD < 6 mm groups as well as PD < 6 mm and control groups, do not indicate statistically significant differences*

Table 3. Tukey's test results. Multiple comparisons of elastase and MMP-9 concentrations in saliva between PD ≥ 6 mm, PD < 6 mm and control groups

Dependent variable	(I) Group 1	(J) Group 1	Mean difference (I-J) [ng/ml]	Standard error	Significance	95% Confidence Interval	
						lower limit	upper limit
elastase	PD ≥ 6 mm	PD < 6 mm	13331.375(*)	2017.478	.000	8429.92	18232.83
		control	15020.740(*)	2130.694	.000	9844.23	20197.25
	PD < 6 mm	PD ≥ 6 mm	−13331.375(*)	2017.478	.000	−18232.83	−8429.92
		control	1689.365	2130.694	.709	−3487.14	6865.87
	control	PD ≥ 6 mm	−15020.740(*)	2130.694	.000	−20197.25	−9844.23
		PD < 6 mm	−1689.365	2130.694	.709	−6865.87	3487.14
MMP-9	PD ≥ 6 mm	PD < 6 mm	293.556	121.169	.051	−.82	587.94
		control	344.596(*)	127.969	.027	33.70	655.50
	PD < 6 mm	PD ≥ 6 mm	−293.556	121.169	.051	−587.94	.82
		control	51.040	127.969	.916	−259.86	361.94
	control	PD ≥ 6 mm	−344.596(*)	127.969	.027	−655.50	−33.70
		PD < 6 mm	−51.040	127.969	.916	−361.94	259.86

Mean difference is significant at 0.05 level

or healthy patients [7, 27-29] as well as increased level of elastase in the course of experimentally induced gingivitis [30]. Furthermore, a reduced level of elastase in gingival crevicular fluid was observed also after surgical and non-surgical treatment [31-33], and in patients treated pharmacologically for rheumatoid arthritis [34].

Studies by Alpagot *et al.* [35] indicate that the level of elastase in gingival crevicular fluid – as well as the patient's age and tobacco smoking – is one of the risk factors for periodontitis in patients with diabetes.

Studies by Wells *et al.* [27] indicated an increased level of elastase in gingival crevicular fluid of patients with periodontitis compared to healthy individuals. Palcanis *et al.* [7] in their research tried to develop a diagnostic test based on measurement of the elastase concentration level in gingival crevicular fluid, which would allow to determine the risk of activity and progress of the disease in given examined sites. The authors proved that elastase concentrations were significantly higher in sites showing progression of periodontal disease (clinical attachment loss and alveolar bone loss during 6 months). The total elastase level in samples of gingival crevicular fluid was assessed by means of spectrofluorometric method. Similar results were obtained by Armitage *et al.* [36]. They demonstrated that examined sites where high elastase levels were observed bear a higher risk of progressive alveolar bone loss, determined by means of digital radiography. Determination of sites with a higher risk of progressive alveolar bone loss would allow to develop a standard treatment procedure for such sites, a procedure that would involve further treatment

without turning to the maintenance phase in such sites, or application of additional e.g. surgical treatment methods, or application of additional chemical substances, e.g. antibiotics administered topically. Patients with such sites would also require more frequent check-up visits. Also Jin *et al.* [37, 38] observed significantly higher elastase levels in gingival crevicular fluid in patients with so-called refractory periodontitis compared to patients with similar progression of periodontitis who responded positively to applied treatment.

Subsequent studies on the role of elastase in destruction of periodontal tissues were based on assessing its activity in gingival crevicular fluid [31, 32, 39]. Eley and Cox [32] demonstrated that elastase activity in gingival crevicular fluid impacts the severity of periodontal disease and is associated with clinical attachment loss. Research by Jin *et al.* [37, 38] proved that activity of granulocytic elastase in gingival crevicular fluid is positively correlated with response to applied treatment of periodontitis. An association between histologically confirmed clinical attachment loss and increased elastase activity was demonstrated by Renvert *et al.* [39] in a study on beagle dogs. Chen *et al.* [40] tried to develop diagnostic tests for assessment of progression and progression risk of periodontal disease based on determining the activity of MMP-8 and elastase in gingival crevicular fluid in patients with chronic periodontitis. Elastase activity was established by means of fluorogenic substrate. The studies showed that both clinical parameters of periodontal tissues and total levels of examined enzymes [ng/sample] in gingival crevicular

Table 4. Correlations of age, number of teeth, elastase and MMP-9 concentrations in the control group and the PD ≥ 6 mm and PD < 6 mm groups

Group			Age	No of teeth	Elastase	MMP-9
Control	Age	Correlation coefficient	1	−.137	−.050	.550
		p		.656	.870	.051
		N	13	13	13	13
	No of teeth	Correlation coefficient	−.137	1	.259	.019
		p	.656		.393	.950
		N	13	13	13	13
	Elastase	Correlation coefficient	−.050	.259	1	.476
		p	.870	.393		.100
		N	13	13	13	13
	MMP-9	Correlation coefficient	.550	.019	.476	1
		p	.051	.950	.100	
		N	13	13	13	13
PD < 6	Age	Correlation coefficient	1	−.763(**)	−.211	−.304
		p		.001	.432	.253
		N	16	16	16	16
	No of teeth	Correlation coefficient	−.763(**)	1	.242	.330
		p	.001		.367	.212
		N	16	16	16	16
	Elastase	Correlation coefficient	−.211	.242	1	.201
		p	.432	.367		.456
		N	16	16	16	16
	MMP-9	Correlation coefficient	−.304	.330	.201	1
		p	.253	.212	.456	
		N	16	16	16	16
PD ≥ 6	Age	Correlation coefficient	1	−.624(**)	−.288	−.297
		p		.010	.280	.265
		N	16	16	16	16
	No of teeth	Correlation coefficient	−.624(**)	1	.491	.411
		p	.010		.054	.114
		N	16	16	16	16
	Elastase	Correlation coefficient	−.288	.491	1	.854(**)
		p	.280	.054		.000
		N	16	16	16	16
	MMP-9	Correlation coefficient	−.297	.411	.854(**)	1
		p	.265	.114	.000	
		N	16	16	16	16

*Correlation is significant at 0.01 level (two-way)

fluid are lowered as a result of non-surgical periodontal treatment. Furthermore, collagenase activity was correlated with gingival index and bleeding index. Amounts of the two examined enzymes were also correlated with each other. Figueredo and Gustafsson [41] demonstrated an increased level of active elastase in gingival crevicular fluid collected from sites indicating significant destruction of periodontal tissues. Jin *et al.* [42, 43] studied a relationship

between elastase activity together with PGE2 level in gingival crevicular fluid and the presence of periopathogenic bacteria (*Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Porphyromonas gingivalis, Prevotella intermedia, Treponema denticola*) in patients with untreated periodontitis. The authors demonstrated that topical immunological response to periopathogens is varied depending on intensity of immunological response measured by the level of elastase and PGE2 in gingival crevicular fluid.

A study by Ingman *et al.* [44] showed that MMP-9 is the main "gelatinase" in gingival crevicular fluid and saliva of patients with adult periodontitis (currently chronic periodontitis). Seguier *et al.* [45] demonstrated a direct relationship between the presence of inflammatory cells on the one hand and the level of selected metalloproteinases and collagen degradation in periodontal tissues on the other hand. Analyzing gingival biopsies of patients with periodontitis and healthy subjects, the authors found that the level of MMP-9 (among other parameters) was significantly higher in the study group than in the control group. Moreover, the level of active MMP-9 was positively correlated with elevated levels of B cells, macrophages and cytotoxic cells, and with the degree of collagen fiber degradation in the examined samples. Numerous studies indicate elevated MMP-9 levels in gingival crevicular fluid of patients with periodontitis compared to healthy subjects [46-49].

The results of quoted studies prove the significance of elastase and MMP-9 in the pathomechanism of periodontitis. In our own study, levels of both enzymes were assessed in saliva, not *in situ* in gingival crevicular fluid. It was assumed that mean concentrations of the studied enzymes in saliva can be resultants of concentrations in individual periodontal pockets. A significantly higher concentration of elastase in saliva in patients with chronic periodontitis compared to healthy individuals ($p < 0.01$) was observed, as well as a statistically significant difference in concentration of MMP-9 in saliva of patients with advanced periodontal disease (PD ≥ 6 mm), as compared to healthy subjects ($p = 0.017$). These results are consistent with results described by Gursay *et al.* [50] who revealed a higher concentration of MMP-9 in saliva of periodontitis patients than in controls. These results support the argument that determining the studied enzymes in saliva can be used to develop a rapid and simple screening method for periodontal disease.

In this study, concentrations of elastase and MMP-9 in saliva in patients with chronic periodontitis and pocket depths PD ≥ 6 mm were compared to concentrations of elastase and MMP-9 in saliva in patients with chronic periodontitis and pocket depths PD < 6 mm. A statistically significant difference in elastase concentrations in saliva which was observed between the group with PD ≥ 6 mm and the group with PD < 6 mm, and also between the group with PD ≥ 6 mm and the control group, can indicate

that periodontal pockets PD ≥ 6 mm are the main source of the increased concentration of the enzyme in saliva. In turn, no statistically significant difference between patients with PD < 6 mm and the control group can indicate that periodontitis diagnosis, which is based solely on clinical attachment loss, may be insufficient, and that the progression of the disease and intensity of inflammation is determined by pocket depth (PD). These results are consistent with Offenbacher *et al.*'s [51] proposed a new approach to the issue of division and diagnostic criteria for periodontal disease according to which patients with PD < 4 mm are classified as healthy (BOP < 10%) or as patients with gingivitis (BOP > 10%).

The lack of statistically significant differences between the levels of MMP-9 in patients with mild periodontal disease (PD < 6 mm) and healthy subjects as well as between groups of patients with different degrees of severity of the disease (PD ≥ 6 mm vs. PD < 6 mm) suggests that elastase should be considered as a more sensitive clinical indicator of the severity of the periodontal disease itself.

This assumption is also confirmed by the positive correlation between MMP-9 and elastase concentration in saliva only in patients with advanced periodontal disease (lack of correlation in the PD < 6 mm group and in the control group). This may mean that the studied enzymes do not affect each other in the site of inflammation, and their concentrations increase only due to the action of the same inflammatory factors that stimulate cells to their increased secretion.

Elastase and MMP-9 concentrations in saliva can be considered as biochemical indicators of severity of periodontitis.

The authors declare no conflict of interest.

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