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ORIGINAL ARTICLE

Characteristics of collagenase-2 from gingival crevicular fluid and peri-implant sulcular fluid in periodontitis and peri-implantitis patients: pilot study

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Abstract

Objective. To compare collagenase activity and collagenolytic matrix metalloproteinase (MMP) levels in gingival crevicular fluid (GCF) and in peri-implant sulcular fluid (PISF) in gingivitis (G), chronic periodontitis (CP), and peri-implantitis (PI) human subjects. ***Material and Methods.*** GCF and PISF were collected on filter paper strips, volume was determined, and samples were extracted in buffer containing general proteinase but not MMP inhibitors. Collagenase activity was measured using a DNP-synthetic octapeptide, and molecular and activation forms of collagenase-2 by Western immunoblotting. ***Results.*** GCF from CP and G sites exhibited elevated collagenase activity and flow, but collagenase concentrations expressed per μl were not significantly different between the healthy and G sites. Minimal fluid was obtained from healthy PISF, and collagenase concentration was the same or lower than in healthy GCF. Although PISF flow was 34% lower than GCF flow in CP subjects, collagenase concentration in CP and in PI sites was 78% and 971% greater, respectively, than in the appropriate healthy sites. Western immunoblot revealed MMP-8 in both PISF and GCF; fibroblast-type MMP-8 was not detected in healthy GCF and PISF. Immunoreactivity level and inactive and activated forms of PMN-type MMP-8 in GCF and PISF increased with the severity of periodontitis and peri-implantitis. Enhanced levels of fibroblast-type MMP-8 in active form were detected only in severe CP GCF and PI PISF. ***Conclusions.*** Peri-implantitis PISF contained higher collagenase-2 levels and activity than GCF from similar deep CP sites. GCF and PISF from severe CP and PI exhibited the highest activation of MMP-8 isoenzymes species (PMN and fibroblast-type).

Key Words: *Collagenase-2, oral fluid, peri-implantitis, periodontitis*

Introduction

Implantology is a rapidly advancing area of dentistry providing a number of alternative treatment modalities for the edentulous and partially edentulous patient for more than 30 years now. The use of implants as abutments for fixed or removable prostheses has significantly increased during the past decade because of their excellent long-term prognosis and prevalence in both esthetics and function. Occasionally, however, dental implant failure still occurs. It is clear that peri-implantitis (PI) associated with bone loss has a negative effect on the long-term prognosis of dental implant reconstruction [1,2]. Researchers have suggested that the soft tissues surrounding an osseo-integrated dental implant bear certain similarities with the periodontium in the natural dentition [3]. A similar bacterial flora is associated with both chronic periodontitis (CP) and PI [4]. Peri-implant sulcular fluid (PISF) has been found to contain host-derived matrix metalloproteinases (MMPs), such as the collagenases, inflammatory cytokines and tissue degradation products [5–10]. However, the diagnosis of PI, as well as its etiology, requires further study. It is still necessary

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to review the knowledge of some characteristics of PI and to study its relationship to CP and to collagenolytic MMPs, which are found to have excessive activity during CP. Accordingly, it is important to determine whether collagenolytic MMPs, as the key enzymes in periodontal ligament breakdown and bone resorption, also play a role in PI [9,11–16].

Increasing evidence indicates that pathologically excessive collagenase activity plays a major role in periodontal destruction [11,12]. This is not surprising, since collagen is the major structural protein of all periodontal tissues [3], and collagenolytic MMP-1, -8, and -13 are the only neutral proteinases that have the ability to initiate the digestion of type I collagen, the most dominant collagen in these periodontal and PI tissue compartments [14]. This led us to explore whether the collagenolytic MMP, i.e. MMP-8, especially since MMP-8 is the most dominant collagenolytic proteinase in inflamed gingiva and GCF, compared to MMP-1 and MMP-13 [12,17], may play a similarly important role in PI [8,9,14].

The aim of this study was to identify whether there are significant differences between the collagenolytic activity in implant recipients with or without PI; and whether increasing the clinical severity of PI is associated with an increase in the levels of collagenolytic activity. We hypothesize that inflammation might affect the levels and degree of activation of MMP-8.

Material and methods

Patients

Human patients, 10 dental implant recipients with either healthy or inflamed PI mucosa (7 F and 3 M) and 19 patients with varying degrees of periodontal disease (9 F and 10 M), aged between 23 and 72 years, were enrolled in the study (Table I). They were recruited from the patient pool of the postgraduate clinics of Periodontics and Prosthodontics at the University of Medicine and Dentistry, New Jersey (UMDNJ). Informed consent was obtained at the first appointment; a medical history was taken and an oral examination carried out. Individuals with the following medical conditions were excluded from the study: people with a medical condition requiring pre-medication prior to dental procedures/visits or currently using antibiotics or host-modulating agents (e.g. Periostat), daily use of NSAIDs or other disease of the oral mucosal or alveolar tissues, pregnant or nursing women, participation in any other clinical study or test panel within the 3 months prior to enrollment in this study, smoking, and immune-compromised individuals (immunosuppressive drug therapy, etc.). Nobel Biocare (Nobel Biocare AB, Göteborg, Sweden) implants were used as implant fixtures and were loaded at least one year before any clinical signs of PI appeared. The protocol for the collection of samples from human subjects was approved by the ethics committees of the School of Dental Medicine, Stony Brook University, Stony Brook, New York, USA and UMDNJ.

Collection of gingival crevicular fluid (GCF) and peri-implant sulcular fluid (PISF) samples and sample preparation

GCF and PISF samples were collected from sample sites as follows: pre-cut sterile filter paper strips (Periopaper; Proflow, Amityville, NY, USA) were inserted for 10 s into the mesial-buccal site of each isolated and air-dried sulcus or pocket until slight resistance was felt; the volume of GCF/PISF collected was determined using a Periotoron 6000 (Proflow, Amityville, NY, USA) as described previously [12,17,18] and samples were immediately placed in microfuge tubes (Sarstedt, Numbrecht, Germany) on ice and transferred to -80°C for storage until processed for analysis of collagenase activity and Western immunoblotting [12,18].

Immediately after the GCF/PISF samples were collected for each patient, the following clinical periodontal parameters of healthy and diseased sites were recorded for each site: plaque index [5,19,20], gingival index [19], and tooth mobility; pocket/probing depth and CAL (clinical attachment level of tooth) were measured using a manual probe (NC-15). Alveolar bone loss was verified by periapical radiographs [21]. Clinical diagnosis of the CP patients was based on the following indices: visible plaque index (VPI), gingival bleeding (GB)

Table I. Patient groups.

Patient group	Number of patients	Age range	Number of female	Number of male
Healthy	4	23–42	0	4
Gingivitis	5	40–50	4	1
Moderate periodontitis	5	40–72	2	3
Severe periodontitis	5	40–72	3	2
Healthy implants	5	50–72	4	1
Peri-implantitis	5	50–72	3	2
Total	29	23–72	16	13

Table II. Characteristics of periodontal and peri-implant sites studied.

Patient group	Gingival Index	Pocket Depth	Tooth Mobility
Healthy gingiva	0	<2 mm	0
Gingivitis	1	2–4 mm	0–2
Moderate periodontitis	1–2	4–6 mm	0–2
Severe periodontitis	3	>6 mm	0–2
Healthy Implants	0	<3 mm	0
Peri-implantitis	3	4–6 mm	0

[22], retentive calculus (RC), pocket-probing depths, and radiographic bone loss detected by orthopantomograms [21]. A total of 89 sample sites were included in this study: healthy gingiva (HG), gingivitis (G) with inflamed gingival tissue, bleeding on probing but no alveolar bone loss around the teeth (pocket depth 2–4 mm), moderate CP (m-CP) with alveolar bone loss (pocket depth range 4–6 mm), severe CP (s-CP) (pocket depth range > 6 mm), healthy dental implants (HI) with no bone loss around implant (pocket depth <3 mm) and PI (pocket depth range from 4 to 6 mm), with inflamed peri-implant mucosa, bleeding on probing, and radiographic alveolar bone loss without mobility (Table II). All samples were collected by one examiner.

Crevicular fluid on periopaper strips was eluted with 400 µL of 50 mmol/l Tris-HCl buffer (pH 7.6) containing a "cocktail" of general proteinase, but not MMP inhibitors, i.e. 1 mg/L antipain, 1 mg/L aprotonin, 1 mg/L leupeptin, and 50 mg/L Zwittergent (Calbiochem-Novabiochem, San Diego, Calif., USA) [3–12], for 1 h at 4°C as described previously [17].

Collagenase activity was measured using DNP-octapeptide containing gly-ileu susceptible peptide bond as substrate [18]. Both the substrate and the tripeptide breakdown product absorb at 380 nm with the same extinction coefficient. GCF extract or enzyme was incubated with 1 mmol substrate solution for 18 h at 37°C. The reaction mixture was stopped by adding the stop-solution (30% acetonitrile in 2 mol acetic acid containing 4 mmol 1, 10-phenonthroline); samples were vortexed and centrifuged for 5 min at 10,000 rpm. An aliquot was injected into HPLC (Waters Alliance 2695 System) for analysis and the percentage of lysis was calculated [18].

Western immunoblotting

Western immunoblot analysis of MMP-8 was carried out using 100 µl of GCF and PISF. Samples were lyophilized in speed-vac, dissolved in Laemmli's buffer [23] (pH 7.0) and heated for 5 min at 100°C. After electrophoresis on 7.5% SDS-PAGE, samples were transferred to nitrocellulose membranes. Non-specific binding was blocked by 5% non-fat dry milk (Valio, Finland). The membranes were incubated using primary rabbit polyclonal antibodies specific for various molecular species of MMP-8 and with alkaline phosphatase conjugated anti-rabbit secondary antibody and then visualized [24–26]. The Western blots were scanned and immuno-reactivities were quantified using an image densitometer and expressed as OD units by a Bio-Rad Model GS-700 Imagining Densitometer using the Analyst™ program [25].

Human PMN and rheumatoid synovial fibroblast culture media were used as positive control for distinct MMP-8 isoenzyme forms [5,24].

Statistical methods

Mean values for relative gingival index, probing depth, GCF flow rate and collagenase activities were calculated using per sample site [27]. Analysis of variance (ANOVA) was performed to analyze a general liner sample group within this study. Dunn's method was used for all pairwise multiple comparisons. Differences were accepted as statistically significant when the probability of a type I error was <5% ($p < 0.05$) [12].

Table III. A comparative clinical and biochemical profiles of gingivitis, moderate and severe chronic peridontitis and peri-implantitis and their healthy controls.

Group of subjects	Number of sites	Gingival index	Probing depth	† Bone loss (x-ray)	Periotron score	µL flow/ 10 sec	Collagenase activity per site‡	Collagenase activity per µL
Healthy gingiva	32	0.6±0.1	3.3±0.15	0/32	33±3	0.2±0.01	1.8±0.1	9.3±0.9
Gingivitis	14	1.4±0.14	3.9±0.3	0/14	89±13	0.5±0.07	3.6±0.6	9.75±2.3
Mod-periodontitis	9	1.6±0.2	*5.6±0.3	9/9	109±15	*0.6±0.08	*8.9±1.9	*16.8±7.4
Sev-periodontitis	11	2.0±0	*5.9±0.4	11/11	176±9	*1.0±0.05	*15.8±3.0	*16.5±2.6
Healthy implants	16	1.0±0	2.4±0.2	0/15	38±6	0.2±0.03	0.4±0.1	2.1±0.5
Peri-implantitis	7	**2.0±0**	***5.0±0.3**	7/7	115±18	*0.66±0.1	*14.4±3.8	*22.5±4.8

‡=%lysis of DNP-peptide/site

† number of bone loss sites showing

*significantly different from healthy control, $p < 0.05$

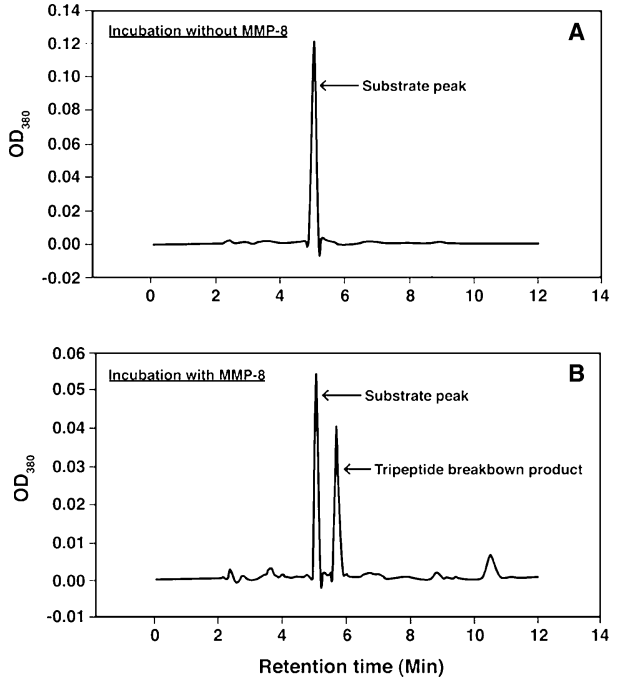

Figure 1. The degradation of DNP-octapeptide by recombinant APMA-activated MMP-8 *in vitro*: detection of tripeptide breakdown product by HPLC. OD 380 indicates optical density red at 380 mn.

Results

Clinical and biochemical profiles of m-CP, s-CP, and PI showed significant increases ($p < 0.05$) compared to healthy gingiva in GCF/PISF flow, probing depth, and collagenase activity, the last-mentioned expressed per site or per µl (Table III). At HG and HI sites, the mean probing depths were 3.3 mm and 2.4 mm, respectively; there was no bleeding on probing detected and no alveolar bone loss observed on periapical radiographic images at these sites. When comparing G to HG sites, the probing depth ranged from 2 to 4 mm; bleeding on probing was detected at some of the G sites and plaque index 2 (data not shown) was found at all sites. However, no bone loss was observed on radiographs. In contrast, for the m-CP, s-CP, and PI sites, the probing depth in general ranged from 4 to 7 mm; there was plaque index 2 in all sites and bleeding upon probing at most sites (Table II). Radiographic alveolar bone resorption was observed within m-CP, s-CP, and PI at all sites recorded. No clinical mobility was found (data not shown). CAL (clinical attachment level) of m-CP and s-CP teeth showed increased levels compared to HG ($p < 0.05$) (data not shown).

GCF and PISF analyses showed elevations of GCF/PISF flow (µl per 10 s) in G, m-CP, and s-CP as well as in PI compared to volume reading of GCF/PISF collected from HG and HI sites ($p < 0.05$) (Table III).

Collagenase activity was assessed (Figure 1) and compared between all six different groups. Our data clearly demonstrated that the collagenase activity per site is high in m-CP, but even higher in s-CP and in PI compared to HG, G, and HI ($p < 0.05$) (Table III). Collagenase activity expressed as a concentration (per µl) was increased substantially more in PI sites than in m-CP and s-CP sites compared to their respective healthy sites (Table III and Figure 2).

Western blot analysis revealed that MMP-8 is present in both healthy and diseased GCF and PISF. Figure 3 further demonstrates that diseased PISF contained more activated and complexed MMP-8 species than diseased GCF. Furthermore, the level of MMP-8 immunoreactivity in PISF and GCF was enhanced with increased clinical severity of both PI and periodontal disease. Also the degree of PMN-type MMP-8 activation was clearly enhanced along with increased clinical severity of both PI and periodontal disease. Fibroblast-type MMP-8 could be detected in partially activated forms only in GCF and PISF from s-CP and PI (Figure 3).

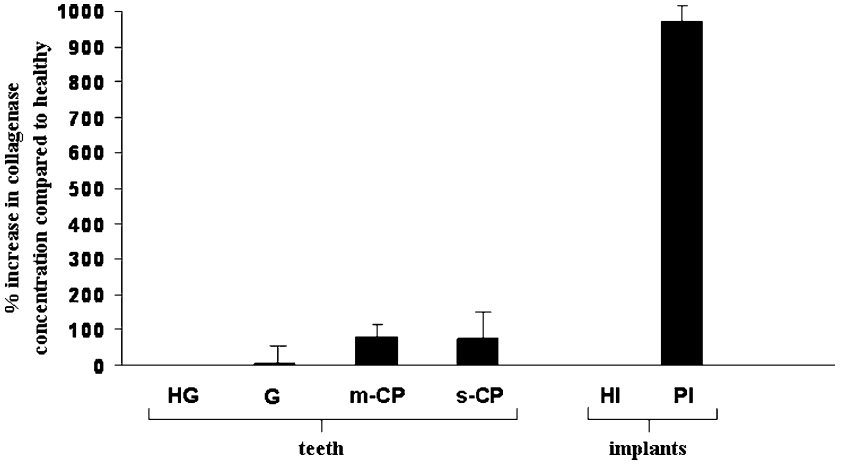

Figure 2. Peri-implantitis increases collagenase concentration in sulcular fluid more than gingivitis and periodontitis. HG = healthy gingiva; G = gingivitis; m-CP = moderate chronic periodontitis; s-CP = severe chronic periodontitis; HI = healthy implant; PI = peri-implantitis.

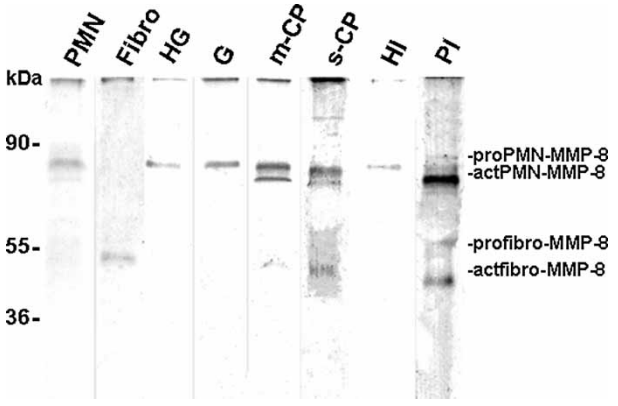

Figure 3. Western immunoblot for molecular forms of MMP-8. PMN = polymorphonuclear leukocyte-type MMP-8; Fibro = fibroblast type-MMP-8; HG = healthy gingiva; G = gingivitis; m-CP = moderate chronic periodonitis; s-CP = severe chronic periodontitis; HI = healthy implant; PI = peri-implantitis. Mobilities of the molecular weight markers are indicated on the left.

Discussion

Our present study revealed that in healthy teeth and dental implant sites, where there is no clinically-detectable inflammation, the GCF and PISF readings and their collagenase activities are barely detectable. In gingivitis, which was not associated with radiographic alveolar bone resorption, the inflammation observed around the gingival margins was accompanied by slightly elevated collagenase activity and MMP-8 immunoreactivity. In moderate and severe CP and in PI sites, accumulated plaque was visible, periodontal probing depth increased, and radiographic alveolar bone resorption could clearly be demonstrated. These clinical findings were accompanied by elevated GCF and PISF readings together with excessive collagenase activities and immunoreactivities for MMP-8, suggesting that the increased enzyme activity could be responsible at least in part for the alveolar bone loss demonstrated by radiographs. A recent study by Golub et al. 2008 [17] supports this concept; they found a strong and statistically significant positive correlation between GCF collagenase activity and a collagen-degradation fragment, a biomarker (ICTP) of bone resorption [17], in pocket sites in a study of 2 years duration. Enhanced immunoreactivity levels and degree of activation of PMN-type MMP-8 were associated with clinical severity of both PI and periodontitis [28,29]. Fibroblast-type isoform of MMP-8 in partially active form could be detected only in PISF and GCF from severe PI and periodontitis. Immunological chair-side tests targeting MMP-8 in GCF have been developed and found to be useful adjunctive diagnostic tools in periodontitis [28,30].

This study suggests that the alveolar bone loss in PI could be directly related to the excessive PISF collagenase activity and activation. Both isoenzyme forms (PMN and fibroblast type) of MMP-8 eventually contribute to this PISF collagenase activity. Noteworthy, the 55 kD fibroblast-type MMP-8 isoform has been shown to be expressed by bone cells [31] and eventually could contribute to the pool of collagenase in PISF and GCF, also to alveolar bone loss. PISF exhibits a potential value for enzyme diagnostics for monitoring the host response in the maintenance phase of dental implant therapy. In conjunction with recent advances in periodontitis treatment/medication and monitoring technology [28,30,32–34] it might allow discrimination of early changes in dental implant's health, and allow early treatment to decrease the failure rate of dental implants. In order to come to a definite conclusion, further studies are needed involving a greater sample size as well as longitudinal not just cross-sectional studies [28,29,34,35].

To conclude, GCF in G, m-CP, and s-CP demonstrated elevated collagenase activity and flow compared to periodontally healthy sites. However, the concentration of collagenase, expressed per μl of GCF, was significantly higher in m-CP, s-CP, and PI sites (80%, 78%, 971%, respectively), compared to HG, G, and HI sites. Western blot analysis indicated that both isoenzyme species of MMP-8 are present in the sulcular fluid around diseased natural teeth and dental implants; the activated/complexed form of MMP-8 was the dominant form of MMP-8 in both GCF and PISF. We acknowledge certain limitations of our study. In our study, the site number is relative low and therefore can only be considered a pilot study [5,7,9,11]. We further suggest that these previous studies together with our present study form a basis for the development of MMP-8 based chair-side diagnostics in peri-implantology [28,30]. Therefore, we are currently further examining the levels of MMP-8 (collagenase-2), known also to be involved in bone resorption [9,31], to be used as adjunctive diagnostic tools [28,30] in the sulcular fluid of CP and PI patients, and the effects of MMP inhibitors (sub-antimicrobial doxycycline) on these parameters [12,18,27,30,32].

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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