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Proteolytic roles of matrix metalloproteinase (MMP)-13 during progression of chronic periodontitis: initial evidence for MMP-13/MMP-9 activation cascade

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Abstract

Aim: Matrix metalloproteinases (MMP)-13 can initiate bone resorption and activate proMMP-9 in vitro, and both these MMPs have been widely implicated in tissue destruction associated with chronic periodontitis. We studied whether MMP-13 activity and TIMP-1 levels in gingival crevicular fluid (GCF) associated with progression of chronic periodontitis assessed clinically and by measuring carboxy-terminal telopeptide of collagen I (ICTP) levels. We additionally addressed whether MMP-13 could potentiate gelatinase activation in diseased gingival tissue.

Materials and Methods: In this prospective study, GCF samples from subjects undergoing clinical progression of chronic periodontitis and healthy controls were screened for ICTP levels, MMP-13 activity and TIMP-1. Diseased gingival explants were cultured, treated or not with MMP-13 with or without adding CL-82198, a synthetic MMP-13 selective inhibitor, and assayed by gelatin zymography and densitometric analysis.

Results: Active sites demonstrated increased ICTP levels and MMP-13 activity ($p < 0.05$) in progression subjects. The MMP-9 activation rate was elevated in MMP-13-treated explants ($p < 0.05$) and MMP-13 inhibitor prevented MMP-9 activation.

Conclusions: MMP-13 could be implicated in the degradation of soft and hard supporting tissues and proMMP-9 activation during progression of chronic periodontitis. MMP-13 and -9 can potentially form an activation cascade overcoming the protective TIMP-1 shield, which may become useful for diagnostic aims and a target for drug development.

Key words: chronic periodontitis progression; ICTP; MMP-9; MMP-13

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Conflict of interest and source of funding statement

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Although chronic periodontitis is an infectious disease initiated by the subgingival microflora, the mediators of connective tissue breakdown are primarily generated by the host's response to the microorganisms (Golub et al. 1997). Destruction of supporting soft and hard tissue including alveolar bone loss is regarded to occur as cycles of acute activity episodes that alternate with prolonged periods of quiescence (Goodson et al. 1984). Collagenases and gelatinases, belonging to the matrix metalloproteinases (MMP) family, eventually play a significant role by means of directly degrading soft tissue and bone collagen (Hill et al. 1994, 1995, Golub et al. 1997). This can be monitored by measuring carboxy-terminal telopeptide of collagen I (ICTP) levels in gingival crevicular fluid (GCF) (Golub et al. 1997). Additionally, increasing interest has recently been focused on proteolytic processing of bioactive non-matrix

substrates by MMPs. In vitro studies have shown that MMP-13 induces proMMP-9 activation and MMP-13 auto-activation (Folgueras et al. 2004). MMP-9 activity, on the other hand, has widely been involved in the pathogenesis of periodontitis and its levels in GCF correlate with clinical parameters (Teng et al. 1992, Pozo et al. 2005, Rai et al. 2008).

Previously, we reported elevated MMP-13 levels in chronic periodontitis and increased MMP-13 activity during disease progression (Hernandez et al. 2006, 2007), strongly suggesting a role for MMP-13 in periodontal soft tissue destruction and/or alveolar bone loss, but the underlying mechanisms are not completely understood.

Pyridinoline cross-linked ICTP is a 12–20 kDa fragment of bone type I collagen released from bone as a result of MMP activity and has been shown to strongly correlate with enhanced bone turnover diseases, such as periodontitis (Giannobile et al. 1995, Golub et al. 1997, Eley & Cox 1998, Al-Shammari et al. 2001, Oringer et al. 2002). Considering that loss of supporting tissues including alveolar bone loss occurs during active episodes of periodontitis, longitudinal studies are required to evaluate the involvement of MMP-13 in direct and indirect periodontal matrix breakdown during episodes of disease progression.

The aims of this study were to determine whether MMP-13 activity and TIMP-1 levels in GCF were associated with progression of chronic periodontitis and periodontal tissue breakdown, assessed clinically and by measuring ICTP levels. We additionally studied whether MMP-13 could potentiate gelatinase activation in periodontitis-affected gingival tissue.

Materials and Methods

Patients and clinical measurements

A longitudinal clinical study was carried out in which moderate to severe chronic periodontitis patients were followed until they developed periodontitis progression. Patients were selected from the Center of Diagnostic and Treatment of Northern Metropolitan Health Services, Santiago and consecutively enrolled. The criteria for entry, as described previously (Hernandez et al. 2006), were a minimum of 14 natural teeth, excluding third molars and including at least 10 posterior teeth. Patients with chronic periodontitis had moderate to advanced periodontitis (at least five to six teeth had sites with probing depth $\geqslant$5 mm with attachment loss $\geqslant$3 mm and extensive bone loss in radiography, according to a classification of the severity of periodontal disease based on the location of the alveolar crest) and had received no periodontal treatment at the time of examination. Subjects did not suffer from systemic illness and had not received antibiotics or non-steroid anti-inflammatory therapy during the 6-month period before the study.

Clinical parameters were evaluated in all teeth, excluding third molars, and included probing depth, clinical attachment loss and dichotomous measurements of supragingival plaque accumulation and bleeding on probing to the base of the crevice (BOP). Six sites were examined for each tooth: mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual. A manual probe (Hu-Friedy, Chicago, IL, USA) was used for attachment level and probing depth. One calibrated examiner monitored the patients and collected the clinical reports.

Disease activity was defined clinically by the tolerance method (Haffajee et al. 1983). At the site level, active sites were defined as those that exhibited attachment loss $\geqslant$2.0 mm during a 2-month period. Inactive sites were defined as those sites with similar probing depth and BOP, but without attachment loss during the same period. At the patient level, at least two active sites were needed to consider the patient as undergoing disease progression.

Measurements of clinical parameters were monitored at baseline, 2 and 4 months and samples were immediately obtained if progression was detected. GCF samples from both active and inactive sites were taken from 21 subjects who underwent disease progression. Upon detection of disease activity, subjects were entered into the treatment phase. Additionally, 11 GCF samples were taken from healthy volunteers.

The protocol was clearly explained to all patients and controls, and Institutional Reviews Board-approved informed consents were signed. The protocol stated that, within 2 weeks from the detection of disease activity, all patients would be provided with periodontal treatment. Periodontal therapy consisted of scaling, root planning and oral hygiene instructions.

Collection of GCF

After isolating the tooth with a cotton roll, supragingival plaque was removed with curettes (Gracey, Hu Friedy), without touching the marginal gingiva. The crevicular site was then dried gently with an air syringe. GCF was collected with paper strips (ProFlow, Amityville, NY, USA) placed into the sulcus/pocket until mild resistance was sensed, and left in place for 30 s (Kiili et al. 2002). Strips contaminated by saliva or blood were excluded from the sampled group. GCF was extracted from the strips by centrifugation at 18,000 *g* for 5 min. at 4°C in 50 μl of elution buffer containing 50 mM Tris HCl pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$ and 0.01% Tritón X 100. The elution procedure was repeated twice, and eluted samples were stored at -80°C until further analysis.

Gingival explant cultures

Gingival tissue biopsies were obtained from inactive sites of periodontitis patients as described previously (Hernandez et al. 2007). Samples consisting of gingival margin, sulcus epithelia and gingival connective tissue were washed extensively in PBS, placed in transport media consisting of DMEM medium and immediately prepared for explant cultures as follows: tissue samples of 40–80 mg were divided into two similar pieces and weighted again, minced and washed with DMEM supplemented with 50 UI/ml penicillin, 50 μg/ml streptomycin and L-glutamine 200 mM, plus fungizone (1.5 mg/ml) (Sigma Chemical Co., St Louis, MI, USA), transferred to a 24-well plate and cultured by adding the same supplemented media at a tissue/media ratio of 100:1 (w/v) in a humidified atmosphere containing 5% CO2 at 37° for 24 h. After testing three different MMP-13 concentrations for 0.5–24 h incubation with the enzyme (not shown), explant cultures were treated with or without recombinant MMP-13 (Chemicon, Temecula, CA, USA) at a 1/3000 ratio (w/v) for 1 h and approximately half of the supernatants of samples and controls per well were recovered, MMP-13 was inactivated with 15 mM EDTA and frozen at -20°. The remaining cultures were further incubated until completion of 24 h and the same procedure was

repeated. Additional controls were made by adding 10 μM CL-82198 (EMD Biosciences, San Diego, CA, USA), a selective synthetic MMP-13 inhibitor, following the manufacturer's recommendations (Fig. 1).

MMP-13 activity measurements

Aliquots of GCF samples were assayed using the ''Fluorokine E'' activity fluorescent assay (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's recommendations. Briefly, specific anti-MMP-13 monoclonal antibodies are pre-coated onto a microplate. GCF aliquots and standards were added to the wells and any MMP-13 is bound to the immobilized antibody. After washing to eliminate any unbound substance, a fluorogenic substrate linked to a quencher molecule was added, and after cleavage by bounded MMP-13, it allows a fluorescent signal proportional to the amount of enzyme activity in the sample. Enzyme activity was expressed as ng of fluorescent product (ng FP) *per* site.

TIMP-1 and ICTP levels in GCF

Periodontitis progression was also screened for ICTP levels as a measurement of bone catabolism. ICTP levels were determined by ''ICTP EIA'' (Orion Diagnostica, Espoo, Finland) and TIMP-1 levels were determined using the ''Biotrack ELISA system'' (Amersham Biosciences, Pittsburg, PA, USA) and following the manufacturer's instructions. Protein levels were obtained from a standard curve and expressed as nanogram per site.

Zymographic assays

Aliquots of supernatants from explants cultures samples and their respective controls were run under non-reducing denaturing conditions, on 10% polyacrylamide gels containing 1 mg/ml gelatin (Merck, Darmstadt, Germany), soaked twice in 2.5% Triton X 100 for 15 min. each and incubated in developing buffer (20 mM Tris pH 7.4 and 5 mM $CaCl_2$) for 17 h. For visualization, gels were stained with Coomassie Brilliant Blue R-250 and destained with 10% acetic acid and 20% methanol solution. Densitometric analysis of gelatinolytic bands was performed using a Bio-Rad Model GS-700 Imaging Densitometer using molecular Analyst™/PC program, and percentage of respective gelatinase activation was calculated as active MMP/(active+total MMP).

Data analysis

In the current study, the values of MMP, ICTP and TIMP were analysed separately using the linear mixed model. The following model was estimated:

$$Y_{ij} = \mu_j + b_i + \varepsilon_{ij},$$

where $i = 1 \ldots 32$ denotes an individual, when $I \leqslant 22$ then group $j = 1$, 2, while for $i > 21$ there is only one observation and group $j = 3$. Further, $b_i \sim N(0, \sigma_b^2)$and $\varepsilon_{ij} \sim N(0, \sigma_j^2)$. For each response, a likelihood ratio test was performed to test the hypothesis that $\sigma_1^2 = \sigma_2^2 = \sigma_3^2$. In each model (MMP, ICTP, TIMP) comparisons between groups were performed with an *F*-test with denominator degrees of freedom computed by the Kenward method; further correction for the multiple testing was applied using Tukey's adjustment.

Differences regarding dichotomic measurements were analysed by the χ^2 test, whereas related or independent comparisons between two groups were performed using a *t*-test (paired and not paired, respectively). Spearman's correlation was applied to determine the association between variables.

The analysis was performed using SAS Proc Mixed (SAS Version 9.1, Copyright © 1999–2001, SAS Institute Inc., Cary, NC, USA).

Results

The clinical features of periodontitis patients *versus* controls are shown in Table 1. Significant differences between the diseased group and controls were observed only with regard to periodontal clinical parameters ($p < 0.05$).

Both MMP-13 activity and ICTP levels increased significantly in active sites compared with inactive and healthy controls, whereas no differences were observed between inactive and healthy sites (Table 2). MMP-13 and ICTP determinations tended to show a negative correlation in active and inactive sites ($r = -0.302$ and -0.09, respectively), whereas healthy sites tended to correlate positively ($r = 0.473$), but none showed significance ($p > 0.05$) (Fig. 2).

On the other hand, TIMP-1 (Table 2) was detected in all controls, but not all progression samples ($p < 0.05$); when detectable levels were measured, there were no differences among progressive sites (active and inactive ones); however, the difference between inactive and healthy sites was borderline non significant ($p = 0.0507$). Dichotomic expression of TIMP-1, as detectable or not, showed increased detection in healthy, followed by inactive and active sites ($p < 0.05$).

Proteolytic activation of gelatinases by addition of MMP-13 was analysed in gingival culture explants from periodontitis patients. Gelatin zymography showed gelatinase expression in all culture supernatants. Bands corresponding to both active and inactive forms of MMP-9 were detected in all MMP-13-treated samples since 0.5–24 h, whereas in controls without MMP-13 and treated with MMP-13 plus CL-82198, a selective synthetic MMP-13 inhibitor, more faint or no active MMP-9 bands were

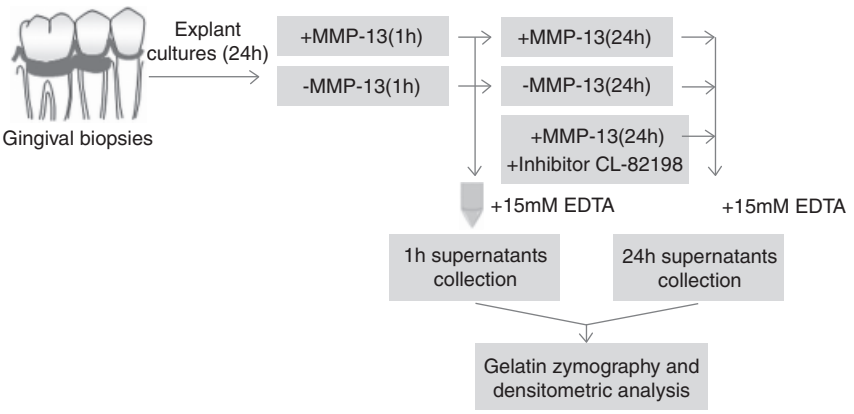

Fig. 1. Diagram representing the sequential steps for diseased gingival tissue preparations.

Table 1. Clinical parameters of progressive periodontitis patients and controls

	Controls ($n = 11$)	**Periodontitis** ($n = 26$)
Age (years)	44.22 ± 6.53	45.90 ± 7.70
Females	8	19
Probing depth (mm)	1.42 ± 0.39	3.65 ± 0.63*
Attachment level (mm)	0.56 ± 0.24	4.02 ± 0.52*
% sites with plaque	17.44	61.78*
% sites with bleeding on probing	7.92	44.56*

Values are expressed as means ± SD.
*$p < 0.0001$.
All p values were determined by t test, except for gender, determined by χ^2 test.

Table 2. MMP-13, ICTP and TIMP-1 determinations in GCF per site

	Active ($n = 21$)	Inactive ($n = 21$)	Controls ($n = 11$)
MMP-13 activity (ng FP)	1.49 ± 0.46*	1.17 ± 0.20	1.03 ± 0.18
ICTP (ng)	0.49 ± 0.21*	0.31 ± 0.15	0.24 ± 0.13
TIMP-1 (% cases)	47.60	76.20	100*
TIMP-1 (ng)	0.319 ± 0.09	0.286 ± 0.10	0.673 ± 0.49

Values expressed as means ± SD. Symbols represent significant differences between groups.
*$p < 0.05$ (p values were determined by ANOVA, excepting TIMP-1 (% cases), determined by χ^2 test. MMP-13 activity: actives *versus* inactives $p = 0.0076$; actives *versus* controls $p = 0.0019$; inactives *versus* controls $p = 0.1351$, ICTP levels: actives *versus* inactives $p = 0.0008$; actives *versus* controls $p = 0.0011$; inactives *versus* controls $p = 0.5908$. TIMP-1 levels: actives *versus* inactives $p = 0.7176$; actives *versus* controls $p = 0.0779$; inactives *versus* controls $p = 0.0507$. TIMP-1 (% of cases), $p = 0.007$.
GCF, gingival crevicular fluid; ICTP, carboxy-terminal telopeptide of collagen I; MMP, matrix metalloproteinases.

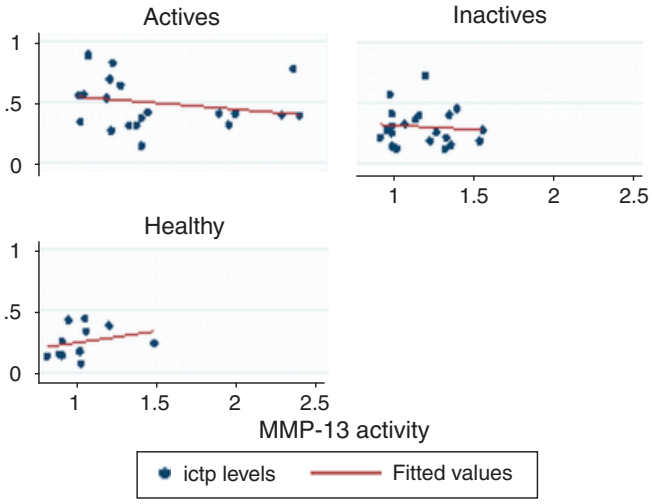

Fig. 2. Correlations between carboxy-terminal telopeptide of collagen I (ICTP) levels and matrix metalloproteinases (MMP-13) activity in gingival crevicular fluid from subjects with progressive chronic periodontitis and controls. Actives: $r = -0.302$; inactives, $r = -0.09$; healthy, $r = 0.473$. $p > 0.05$.

recognized (Fig. 3). MMP-2 active and proforms were also visualized, but they were not present in all analysed samples (not shown). As shown in Table 3, MMP-13-treated gingival culture explants showed increased proMMP-9 activation expressed as percentage of activation *versus* controls, at both 1 and 24 h ($p < 0.05$). Besides, the rate of MMP-9 activation was higher at 24 h compared with 1 h of treatment with MMP-13 ($p = 0.04$), while no differences were found among controls ($p > 0.05$). Similarly, MMP-2 showed a tendency towards an increase in its activation rate after treatment with MMP-13 ($p > 0.05$) compared with controls. Spearman's correlation analysis demonstrated a strong positive correlation among active forms of MMP-9 and MMP-2 in all the groups studied ($r = 0.84$, $p = 0.0000$). Additional faint bands at 48 kDa corresponding to active MMP-13 could be detected in samples at 1 h and tended to disappear towards 24 h (not shown).

Discussion

MMPs have been identified in various tissue destructive diseases including chronic periodontitis, where they have been assumed to primarily play a matrix-degradative role. Furthermore, a broader substrate degradome shows proteolytic susceptibitity to MMPs, including cytokines, chemokines and other MMPs modulating the inflammatory response (McQuibban et al. 2002). In this study, MMP-13 proteolytic activity and its inhibitor TIMP-1 were screened during progression of periodontal disease. Progression was determined clinically, and further molecular characterization was performed by measuring ICTP levels. We also addressed whether MMP-13 could enhance gelatinase activation in periodontitis-affected gingival tissue to elucidate further mechanisms involving MMP-13, besides direct extracellular matrix breakdown. Our results support and further extend the conjuncture that MMP-13 activity could be implicated in the progression of chronic periodontitis by means of periodontal tissue breakdown including alveolar bone resorption, as reflected by higher ICTP levels in active sites and further, by processing of bioactive substrates, resulting in MMP-9 activation.

MMP-13 expression has previously been shown to increase in gingival tissue and GCF from periodontitis subjects (Kiili et al. 2002, Hernandez et al. 2006, 2007) and to correlate with clinical parameters (Tervahartiala et al. 2000) and collagen loss (Ejeil et al. 2003, Uitto et al. 2003); thus, MMP-13 is considered to play a substantial role in periodontal matrix degradation (Ilgenli et al. 2006), but the mechanisms of how MMP-13 takes part in soft or hard tissue hydrolysis is yet to be known.

In this study, we report higher MMP-13 activity and ICTP levels in active sites from progressive periodontitis patients, compared with inactive sites and healthy individuals. Further, we

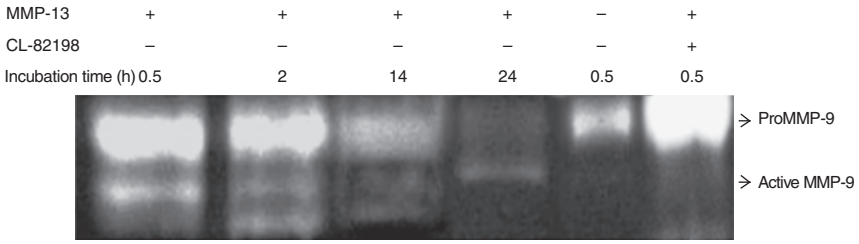

Fig. 3. Pro-matrix metalloproteinases (MMP)-9 activation in MMP-13-treated gingival tissue explants and controls from chronic periodontitis patients. Explants from diseased gingiva were cultured for 24 h and MMP-13 was added to the media for 0.5, 2, 14 and 24 h. Controls without adding MMP-13 and adding MMP-13 plus CL-82198 are also shown. Gelatinolytic bands were visualized by gelatin zymography.

Table 3. Mean activation percentages of MMP-9 and MMP-2 in diseased gingival explants treated or not with MMP-13

	− MMP13 1 h	+MMP13 1 h	*p*	− MMP13 24 h	+MMP13 24 h	*p*
MMP-9	0.160 ± 0.77	0.31 ± 0.94	0.0001	0.210 ± 0.16	0.380 ± .15	0.0014
MMP-2	0.46 ± 0.14	0.49 ± 0.12	0.18	0.51 ± 0.16	0.54 ± 0.15	0.10

Values expressed as means ± SD. − MMP13 1 h, untreated controls, 1 h of incubation; +MMP13 1 h, MMP-13-treated samples, 1 h of incubation; − MMP13 24 h, Untreated controls, 14 h of incubation; +MMP13 24 h, MMP-13-treated samples, 24 h of incubation.
MMP, matrix metalloproteinases.

demonstrate an association between ICTP levels, assumed to be mainly generated from bone collagen by MMPs (Fuller et al. 2007), and disease activity determined clinically by the tolerance method (Haffajee et al. 1983). On the other hand, TIMP-1 levels remained unchanged, suggesting the existence of an imbalance between MMP-13 activity and its major endogenous inhibitor that could result in attachment and alveolar bone loss.

Although many biomarkers have been proposed in chronic periodontitis, most of them demonstrate limited usefulness because they rather reflect inflammation of periodontal tissue than disease progression (Loos & Tjoa 2005, Sorsa et al. 2006). MMP-13 activity could thus represent a marker of disease progression, and to some extent, alveolar bone loss. It has shown to be involved in initiation of bone resorption by removing organic bone matrix and generating collagen fragments that could activate osteoclasts (Holliday et al. 1997). However, we failed to show a direct correlation between MMP-13 activity and ICTP levels. On the one hand, this finding could rely on the fact that ICTP release depends on the activity of many other bone secreted enzymes (Hill et al. 1994); on the other, MMP-13 in GCF is secreted from many cellular sources, which include those from inflamed gingival tissue and thus, it is eventually not specific to the bone (Kiili et al. 2002, Hernandez et al. 2006, 2007).

MMP-13 increments in chronic periodontitis seem to involve all MMP-13 forms: proenzyme, active and fragments (Ilgenli et al. 2006). In the present report, we analysed gingival culture explants of gingival tissue from chronic periodontitis patients treated or not with MMP-13 to elucidate additional potential mechanisms involved in progression of inactive sites to actives. Faint MMP-13 active 48 kDa bands were detected by gelatin zymography, which tended to disappear at 24 h of enzyme incubation. Interestingly, recombinant MMP-13 was not exogenously activated, and so it might be activated by other MMPs secreted by inflamed gingival tissue, which include MMP-14, MMP-2 (Knauper et al. 1996) and MMP-9 (Overall 2002), as well as reactive oxygen species (Ilgenli et al. 2006).

In addition to MMP-13 extracellular matrix-degradative properties, bioactive substrates have been described in vitro, as proMMP-9 activation and self-proteolytic cleavage of proMMP-13 (Knauper et al. 1996, 1997a, Folgueras et al. 2004). We found that the rate of MMP-9 activation increased in MMP-13-treated samples *versus* controls, suggesting that MMP-13 might act as an effective direct or indirect activator of proMMP-9 during chronic periodontitis progression. Further, MMP-9 has previously been described to activate proMMP-13 and proMMP-2 in vitro (Overall 2002, Folgueras et al. 2004), representing an amplification cascade that could perpetuate tissue destruction. In the present study, MMP-2 activation tended to increase in MMP-13-treated samples, whereas active MMP-2 forms increased together with active MMP-9, suggesting that an MMP cascade involving MMP-13, -9 and -2 might be implicated in the pathogenesis of chronic periodontitis in vivo. The mechanisms involved in MMP activation may vary depending on the tissue type and disease. Previous studies in skin showed that proMMP-9 activation is insensible to MMP activities, but induced by chymotrypsin, whereas in hepatic stellate cells, proMMP-9 is activated by MMP-13 (Han et al. 2007). ProMMP-13 in turn can also be initially activated by serine proteases (Sorsa et al. 1992, Moilanen et al. 2003), and with respect to periodontal inflammation, proteases from potent periodontopathogens can/may also activate proMMPs (Sorsa et al. 1992). Several studies have previously addressed the relationship between MMP-9 levels and/or activity in GCF and chronic periodontitis, showing its correlation with clinical parameters, including recurrent attachment loss (Teng et al. 1992, Pozo et al. 2005, Soder et al. 2006, Rai et al. 2008). MMP-9 has further been involved in bone resorption in vitro, playing a role in the subsequent digestion of denatured collagen I after being cleaved by collagenase (Hill et al. 1995). Previous studies based on induced experimental periodontitis in mice have demonstrated elevated levels of MMP-2, -9, -1 and RANKL that correlated with the expression of IL-1β, TNF-α and IF-γ during alveolar bone loss (Garlet et al. 2006). Additionally, MMP-9 activity is thought to act over preosteoclast recruitment to the bone tissue and migration within the marrow to sites for osteoclast differentiation and bone resorption (Yu et al. 2003). Conversely, recent animal model studies have demonstrated that MMP-8 can exert even protective or anti-inflammatory properties against *Porphyromonas gingivalis*-induced periodontitis (Kuula et al. 2009).

In this report, we demonstrate that active sites from patients undergoing progressive chronic periodontitis, characterized by increased periodontal extracellular matrix breakdown, showed higher MMP-13 activity. Because in vitro assays do not replicate necessarily in vivo conditions and are often not performed in the presence of extracellular matrix components that may modulate protease activity (McQuibban et al. 2002), we contribute for the first time with preliminary evidence of in vivo activation of proteolytic cascades of MMP-9 due to the MMP-13 activity related to chronic periodontitis. Previous in vitro studies have shown that MMP-13 can directly cleave proMMP-9 (Knauper et al. 1997b), but further studies should be conducted to clarify the mechanism resulting in increased MMP-9 activation by MMP-13 in periodontal disease. We also demonstrated that a synthetic selective MMP-13 inhibitor could prevent MMP-13-mediated proMMP-9 activation in periodontitis-affected gingival explant cultures, suggesting this cascade as a suitable target for future drug development (Golub et al. 1998, Sorsa et al. 2004). Supporting this, Golub et al. (1997) have previously demonstrated that treatment of chronic periodontitis with low-dose doxycycline reduced alveolar bone loss, ICTP levels and collagenase activity in GCF; furthermore, among collagenases, MMP-13 was reduced even more substantially than MMP-8. Additionally, MMP-13-MMP-9 activation cascade may be utilized for development of chair-side point of care diagnostics for periodontitis (Mantyla et al. 2003, 2006, Sorsa et al. 2004, 2006). Thus, processing of bioactive substrates by MMP-13 could represent a key mechanism of amplification of periodontal tissue destruction and modulation of inflammatory response during chronic periodontitis.

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References

Al-Shammari, K. F., Giannobile, W. V., Aldredge, W. A., Iacono, V. J., Eber, R. M., Wang, H. L. & Oringer, R. J. (2001) Effect of non-surgical periodontal therapy on C-telopeptide pyridinoline cross-links (ICTP) and interleukin-1 levels. *Journal of Periodontology* **72**, 1045–1051.

Ejeil, A. L., Igondjo-Tchen, S., Ghomrasseni, S., Pellat, B., Godeau, G. & Gogly, B. (2003) Expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in healthy and diseased human gingiva. *Journal of Periodontology* **74**, 188–195.

Eley, B. M. & Cox, S. W. (1998) Advances in periodontal diagnosis. 10. Potential markers of bone resorption. *British Dental Journal* **184**, 489–492.

Folgueras, A. R., Pendas, A. M., Sanchez, L. M. & Lopez-Otin, C. (2004) Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. *International Journal of Developmental Biology* **48**, 411–424.

Fuller, K., Kirstein, B. & Chambers, T. J. (2007) Regulation and enzymatic basis of bone resorption by human osteoclasts. *Clinical Science (London)* **112**, 567–575.

Garlet, G. P., Cardoso, C. R., Silva, T. A., Ferreira, B. R., Avila-Campos, M. J., Cunha, F. Q. & Silva, J. S. (2006) Cytokine pattern determines the progression of experimental periodontal disease induced by Actinobacillus actinomycetemcomitans through the modulation of MMPs, RANKL, and their physiological inhibitors. *Oral Microbiology and Immunology* **21**, 12–20.

Giannobile, W. V., Lynch, S. E., Denmark, R. G., Paquette, D. W., Fiorellini, J. P. & Williams, R. C. (1995) Crevicular fluid osteocalcin and pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP) as markers of rapid bone turnover in periodontitis. A pilot study in beagle dogs. *Journal of Clinical Periodontology* **22**, 903–910.

Golub, L. M., Lee, H. M., Greenwald, R. A., Ryan, M. E., Sorsa, T., Salo, T. & Giannobile, W. V. (1997) A matrix metalloproteinase inhibitor reduces bone-type collagen degradation fragments and specific collagenases in gingival crevicular fluid during adult periodontitis. *Inflammation Research* **46**, 310–319.

Golub, L. M., Lee, H. M., Ryan, M. E., Giannobile, W. V., Payne, J. & Sorsa, T. (1998) Tetracyclines inhibit connective tissue breakdown by multiple non-antimicrobial mechanisms. *Advances in Dental Research* **12**, 12–26.

Goodson, J. M., Haffajee, A. D. & Socransky, S. S. (1984) The relationship between attachment level loss and alveolar bone loss. *Journal of Clinical Periodontology* **11**, 348–359.

Haffajee, A. D., Socransky, S. S. & Goodson, J. M. (1983) Comparison of different data analyses for detecting changes in attachment level. *Journal of Clinical Periodontology* **10**, 298–310.

Han, Y. P., Yan, C., Zhou, L., Qin, L. & Tsukamoto, H. (2007) A matrix metalloproteinase-9 activation cascade by hepatic stellate cells in trans-differentiation in the three-dimensional extracellular matrix. *The Journal of Biological Chemistry* **282**, 12928–12939, doi:M700554200 [pii] 10.1074/jbc.M700554200.

Hernandez, M., Martinez, B., Tejerina, J. M., Valenzuela, M. A. & Gamonal, J. (2007) MMP-13 and TIMP-1 determinations in progressive chronic periodontitis. *Journal of Clinical Periodontology* **34**, 729–735.

Hernandez, M., Valenzuela, M. A., Lopez-Otin, C., Alvarez, J., Lopez, J. M., Vernal, R. & Gamonal, J. (2006) Matrix metalloproteinase-13 is highly expressed in destructive periodontal disease activity. *Journal of Periodontology* **77**, 1863–1870.

Hill, P. A., Docherty, A. J., Bottomley, K. M., O'Connell, J. P., Morphy, J. R., Reynolds, J. J. & Meikle, M. C. (1995) Inhibition of bone resorption in vitro by selective inhibitors of gelatinase and collagenase. *The Biochemical Journal* **308** (Part 1), 167–175.

Hill, P. A., Murphy, G., Docherty, A. J., Hembry, R. M., Millican, T. A., Reynolds, J. J. & Meikle, M. C. (1994) The effects of selective inhibitors of matrix metalloproteinases (MMPs) on bone resorption and the identification of MMPs and TIMP-1 in isolated osteoclasts. *Journal of Cell Science* **107** (Part 11), 3055–3064.

Holliday, L. S., Welgus, H. G., Fliszar, C. J., Veith, G. M., Jeffrey, J. J. & Gluck, S. L. (1997) Initiation of osteoclast bone resorption by interstitial collagenase. *Journal of Biological Chemistry* **272**, 22053–22058.

Ilgenli, T., Vardar-Sengul, S., Gurkan, A., Sorsa, T., Stackelberg, S., Kose, T. & Atilla, G. (2006) Gingival crevicular fluid matrix metalloproteinase-13 levels and molecular forms in various types of periodontal diseases. *Oral Disease* **12**, 573–579.

Kiili, M., Cox, S. W., Chen, H. Y., Wahlgren, J., Maisi, P., Eley, B. M., Salo, T. & Sorsa, T. (2002) Collagenase-2 (MMP-8) and collagenase-3 (MMP-13) in adult periodontitis: molecular forms and levels in gingival crevicular fluid and immunolocalisation in gingival tissue. *Journal of Clinical Periodontology* **29**, 224–232.

Knauper, V., Cowell, S., Smith, B., Lopez-Otin, C., O'Shea, M., Morris, H., Zardi, L. & Murphy, G. (1997a) The role of the C-terminal domain of human collagenase-3 (MMP-13) in the activation of procollagenase-3, substrate specificity, and tissue inhibitor of metalloproteinase interaction. *Journal of Biological Chemistry* **272**, 7608–7616.

Knauper, V., Smith, B., Lopez-Otin, C. & Murphy, G. (1997b) Activation of progelatinase B (proMMP-9) by active collagenase-3 (MMP-13). *European Journal of Biochemistry* **248**, 369–373.

Knauper, V., Will, H., Lopez-Otin, C., Smith, B., Atkinson, S. J., Stanton, H., Hembry, R. M. & Murphy, G. (1996) Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase a (MMP-2) are able to generate active enzyme. *Journal of Biological Chemistry* **271**, 17124–17131.

Kuula, H., Salo, T., Pirila, E., Tuomainen, A. M., Jauhiainen, M., Uitto, V. J., Tjaderhane, L., Pussinen, P. J. & Sorsa, T. (2009)

Local and systemic responses in matrix metalloproteinase 8-deficient mice during Porphyromonas gingivalis-induced periodontitis. *Infection and Immunity* **77**, 850–859, doi:IAI.00873-08 [pii] 10.1128/IAI.00873-08.

Loos, B. G. & Tjoa, S. (2005) Host-derived diagnostic markers for periodontitis: do they exist in gingival crevice fluid? *Periodontology 2000* **39**, 53–72.

Mantyla, P., Stenman, M., Kinane, D., Salo, T., Suomalainen, K., Tikanoja, S. & Sorsa, T. (2006) Monitoring periodontal disease status in smokers and nonsmokers using a gingival crevicular fluid matrix metalloproteinase-8-specific chair-side test. *Journal of Periodontal Research* **41**, 503–512, doi:JRE897 [pii] 10.1111/j.1600-0765.2006.00897.x.

Mantyla, P., Stenman, M., Kinane, D. F., Tikanoja, S., Luoto, H., Salo, T. & Sorsa, T. (2003) Gingival crevicular fluid collagenase-2 (MMP-8) test stick for chair-side monitoring of periodontitis. *Journal of Periodontal Research* **38**, 436–439, doi:677 [pii].

McQuibban, G. A., Gong, J. H., Wong, J. P., Wallace, J. L., Clark-Lewis, I. & Overall, C. M. (2002) Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood* **100**, 1160–1167.

Moilanen, M., Sorsa, T., Stenman, M., Nyberg, P., Lindy, O., Vesterinen, J., Paju, A., Konttinen, Y. T., Stenman, U. H. & Salo, T. (2003) Tumor-associated trypsinogen-2 (trypsinogen-2) activates procollagenases (MMP-1, -8, -13) and stromelysin-1 (MMP-3) and degrades type I collagen. *Biochemistry* **42**, 5414–5420, doi:10.1021/bi020582s.

Oringer, R. J., Al-Shammari, K. F., Aldredge, W. A., Iacono, V. J., Eber, R. M., Wang, H. L., Berwald, B., Nejat, R. & Giannobile, W. V. (2002) Effect of locally delivered minocycline microspheres on markers of bone resorption. *Journal of Periodontology* **73**, 835–842.

Overall, C. M. (2002) Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exosites. *Molecular Biotechnology* **22**, 51–86, doi:MB:22:1:051 [pii] 10.1385/MB:22:1:051.

Pozo, P., Valenzuela, M. A., Melej, C., Zaldivar, M., Puente, J., Martinez, B. & Gamonal, J. (2005) Longitudinal analysis of metalloproteinases, tissue inhibitors of metalloproteinases and clinical parameters in gingival crevicular fluid from periodontitis-affected patients. *Journal of Periodontal Research* **40**, 199–207.

Rai, B., Kharb, S., Jain, R. & Anand, S. C. (2008) Biomarkers of periodontitis in oral fluids. *Journal of Oral Science* **50**, 53–56, doi:JST.JSTAGE/josnusd/50.53 [pii].

Soder, B., Airila Mansson, S., Soder, P. O., Kari, K. & Meurman, J. (2006) Levels of matrix metalloproteinases-8 and -9 with simultaneous presence of periodontal pathogens in gingival crevicular fluid as well as matrix metalloproteinase-9 and cholesterol in blood. *Journal of Periodontal Research* **41**, 411–417, doi:JRE888 [pii] 10.1111/j.1600-0765.2006.00888.x.

Sorsa, T., Ingman, T., Suomalainen, K., Haapasalo, M., Konttinen, Y. T., Lindy, O., Saari, H. & Uitto, V. J. (1992) Identification of proteases from periodontopathogenic bacteria as activators of latent human neutrophil and fibroblast-type interstitial collagenases. *Infection and Immunity* **60**, 4491–4495.

Sorsa, T., Tjaderhane, L., Konttinen, Y. T., Lauhio, A., Salo, T., Lee, H. M., Golub, L. M., Brown, D. L. & Mantyla, P. (2006) Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Annals of Medicine* **38**, 306–321, doi:W1Q8Q10353707646 [pii] 10.1080/07853890600800103.

Sorsa, T., Tjaderhane, L. & Salo, T. (2004) Matrix metalloproteinases (MMPs) in oral diseases. *Oral Disease* **10**, 311–318, doi:ODI1038 [pii] 10.1111/j.1601-0825.2004.01038.x.

Teng, Y. T., Sodek, J. & McCulloch, C. A. (1992) Gingival crevicular fluid gelatinase and its relationship to periodontal disease in human subjects. *Journal of Periodontal Research* **27**, 544–552.

Tervahartiala, T., Pirila, E., Ceponis, A., Maisi, P., Salo, T., Tuter, G., Kallio, P., Tornwall, J., Srinivas, R., Konttinen, Y. T. & Sorsa, T. (2000) The in vivo expression of the collagenolytic matrix metalloproteinases (MMP-2, -8, -13, and -14) and matrilysin (MMP-7) in adult and localized juvenile periodontitis. *Journal of Dental Research* **79**, 1969–1977.

Uitto, V. J., Overall, C. M. & McCulloch, C. (2003) Proteolytic host cell enzymes in gingival crevice fluid. *Periodontology 2000* **31**, 77–104.

Yu, X., Collin-Osdoby, P. & Osdoby, P. (2003) SDF-1 increases recruitment of osteoclast precursors by upregulation of matrix metalloproteinase-9 activity. *Connective Tissue Research* **44** (Suppl. 1), 79–84.

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Clinical Relevance

Scientific rationale for the study: MMP-13 has previously been involved in active episodes of attachment loss in chronic periodontitis, but the underlying mechanisms are not fully clarified.

Principal findings: MMP-13 activity was significantly elevated in active sites, together with high levels of ICTP, showing an association with progression of periodontal breakdown. Furthermore, MMP-13-enhanced MMP-9 activation may promote tissue destruction during chronic periodontitis.

Practical implications: MMP-13 together with MMP-9 could represent useful biomarkers for periodontitis progression and targets for future drug development.