Matrix Metalloproteinase-13 Is Highly Expressed in Destructive Periodontal Disease Activity

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Background: Matrix metalloproteinases (MMPs) participate in extracellular matrix degradation in physiological and pathological conditions. The available evidence suggests that MMP-13 plays a significant role in both the initiation and progress of bone resorption. The aim of our study was to identify the presence of MMP-13 in adult patients with untreated chronic periodontitis. We also determined the activity of MMP-13 present in lesions undergoing episodic attachment loss in gingival crevicular fluid (GCF) samples.

Methods: After monitoring at 2 and 4 months, 21 patients showed destructive periodontitis (periodontally affected sites presenting at least two sites with ≥2 mm clinical attachment loss), and GCF samples were collected both from active and inactive sites (21 GCF samples, each). GCF was collected during a 30-second interval using a paper strip, and an immunofluorescence assay was performed to determine the basal activity of MMP-13 and the relationship between 4-aminophenylmercuric acetate (APMA)-activated total MMP-13 and basal MMP-13 activity. Gingival tissues from five patients were fixed in formalin and MMP-13 expression was demonstrated using immunohistochemistry and *in situ* hybridization. MMP-13 molecular forms were examined by Western immunoblotting with monoclonal antibodies.

Results: MMP-13 was found in 100% of GCF samples from patients with chronic periodontitis. Active sites, associated with tissue destruction, had significantly higher proportions of active MMP-13 and MMP-13 activity levels than their inactive counterparts (1.49 versus 1.17 ng fluorescent product, respectively; $P<0.05$). Western blot, immunohistochemical staining, and *in situ* hybridization confirmed the presence of MMP-13 in periodontal disease, with observable differences between periodontitis and healthy subjects. MMP-13 immunoreactivities were seen mainly as 55 and 48 kDa, corresponding to partially and fully activated forms, respectively, and a smaller proportion of 60-kDa proenzyme form.

Conclusion: MMP-13 activity in GCF samples was significantly increased in active sites from progressive periodontal disease, supporting its role in the alveolar bone loss developed in this disease. *J Periodontol 2006;77:1863-1870.*

KEY WORDS

Collagenase-3; gingival crevicular fluid; matrix metalloproteinase-13; periodontal disease; periodontitis.

Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth caused by groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession, or both.[1] Destruction of the osseous support of the dentition is a hallmark of chronic periodontitis.[2] The tissue destruction appears to result from a complex interaction between these bacteria and the host's immune and inflammatory responses. Proteolytic activities from these bacteria, including collagenase, may participate in collagen degradation.[3] Activation and overexpression of host matrix metalloproteinases (MMPs) caused by periodontal pathogens like *Actinobacillus gingivalis* and *Porphyromonas gingivalis* and by inflammatory cytokines have been reported.[4-8] Interstitial collagenase activities in gingival tissue extracts and gingival crevicular fluid (GCF) from periodontitis sites are pathologically elevated compared to the periodontally healthy gingival tissue and GCF.[9-13]

MMPs are a family of proteolytic enzymes involved in the

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physiological degradation of extracellular matrix proteins and basement membrane.[14,15] Historically, MMPs were divided into several subgroups of MMPs: collagenases (MMP-1, - 8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), and membrane-type associated MMPs.[15] However, as the list of MMP substrates have grown, a sequential numbering system for the MMPs has been adapted, and the MMPs are now grouped according to their structure.[16] MMP-13 (collagenase-3) has been considered to have an important role in skeletal biology in view of its exclusive presence in the skeleton during embryonic development in cartilaginous growth plates and primary centers of ossification.[17-19]

MMP-13 has been shown to be expressed by periodontitis-affected human gingival sulcular epithelium[20] and gingival fibroblasts,[21] but its role in destructive periodontal disease activity is not fully understood. The detection of MMP-13 activity in the GCF of periodontitis patients with lesions undergoing episodic attachment loss could partially explain the mechanisms associated with the destruction of the supporting tissues of the tooth. We determined the presence and molecular forms of MMP-13 in gingival tissue biopsies and GCF, respectively, from patients diagnosed with chronic periodontitis and healthy subjects. We measured MMP-13 activity in GCF samples from active sites where bone tissue destruction clearly occurred and compared these samples with inactive sites from patients undergoing progression of chronic periodontitis.

MATERIALS AND METHODS

Patients
This was a longitudinal clinical study in which moderate to severe chronic periodontitis patients were followed until they developed periodontitis progression. The 76 patients were selected from the Center of Diagnosis and Treatment, Northern Metropolitan Health Services, Santiago, Chile, and consecutively enrolled between October 2004 and July 2005. The criteria for entry were ≥14 natural teeth, excluding third molars, and including ≥10 posterior teeth. Patients with chronic periodontitis had moderate to advanced periodontitis (at least five or six teeth had sites with probing depths (PDs) ≥5 mm, attachment loss ≥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 not received periodontal treatment at the time of examination. Subjects did not have a systemic illness and had not received antibiotics or nonsteroid anti-inflammatory therapy during the 6-month period prior to the study. Prior to the study, all subjects received supragingival prophylaxis to remove gross calculus to allow the measurement of PD. The protocol was clearly explained to all patients and controls, and institutional review board–approved informed consents were signed. The protocol stated that, within 2 weeks of the detection of disease activity, all patients would be provided with periodontal treatment. Periodontal therapy consisted of scaling and root planing and oral hygiene instructions.

Clinical Measurements
Clinical parameters were evaluated in all teeth, excluding third molars, and included: PD, clinical attachment loss (CAL), and dichotomous measurements of supragingival plaque accumulation (plaque index [PI]), and bleeding on probing (BOP) to the base of the crevice. Six sites were examined for each tooth: mesio-buccal, buccal, disto-buccal, disto-lingual, lingual, and mesio-lingual. An automated disk probe was used for CAL and PD.§ One calibrated examiner monitored the patients and collected the clinical reports.

Attachment level and PD data were taken again 7 days after the beginning of the study. Disease activity was defined by the tolerance method.[22] At the site level, active sites were considered if they exhibited attachment loss ≥2.0 mm during the following 2-month period. Inactive sites were defined as those sites with probing depth and BOP equivalent to active sites, 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. Clinical parameter measurements were monitored at baseline and 2 and 4 months, and GCF collections were performed at 2 or 4 months from one active site and one inactive site simultaneously on each patient undergoing progression before the periodontal therapy was carried out. Nine patients (nine of 76 patients; 11.84%) exhibited disease activity during the first 2 months, and 12 patients exhibited disease activity during the following 4-month period (12 of 76 patients; 16.78%). A total of 21 subjects underwent disease progression from the total of 76 chronic periodontitis patients examined, and thus they were included in this study. Upon the detection of disease activity, subjects were entered into the treatment phase.

GCF samples were collected from one active site and one inactive site from the 21 patients demonstrating disease activity, as soon as activity was detected, and MMP-13 levels from active (21 samples) and inactive (21 samples) sites were compared.

Immunohistochemistry
Gingival tissue biopsies from patients diagnosed with chronic periodontitis and from healthy subjects were obtained under local anesthesia. Incisions of the

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inflamed tissues were made 1 to 2 mm subgingivally. Sections were fixed in 4% paraformaldehyde at 4°C overnight. Tissue sections of 7µ were obtained and deparaffined, and endogenous peroxidase activity was blocked with 10% hydrogen peroxide for 10 minutes. After washing, non-specific tissue binding was blocked by incubation with 1% horse serum for 30 minutes at room temperature and washed again. Specimens were incubated overnight with 1:500 diluted primary MMP-13 monoclonal antibody∥ in Tris-buffered saline and 0.1% Tween 20 (TBST 0.1%). All rinsing steps were performed with TBST 0.1%, pH 7.2 to 7.6. After washing, goat anti-mouse immunoglobulin G (IgG) secondary antibodies conjugated with peroxidase were used at a 1:200 dilution for 30 minutes. A brown color was developed by exposure for 3,3'-diaminobenzidine tetrahydrochloride (DAB).¶ Sections where counterstained with hematoxylin,# dehydrated, and permanently mounted. Positive and negative controls were processed with each series.

In Situ *Hybridization*

In situ hybridization was performed as previously reported.[23] Sections were hybridized with [^{35}S]-labeled antisense riboprobes and subsequently exposed to photographic emulsion at 4°C for several days, developed, fixed, cleared, and counterstained with 0.02% toluidine blue. Sections hybridized with a labeled-sense riboprobe were used as negative controls. Either sense or antisense [^{35}S] uridine triphosphate–labeled RNA probes were synthesized from the corresponding linearized DNA using the appropriate RNA polymerases. Probes for *in situ* hybridization were as follows: probe for mouse rat collagenase-3 was a 314-base pair (bp) fragment corresponding to nucleotides 350 to 653 of the rat collagenase-3 gene (GenBank accession no. M60616).

Western Immunoblot

MMP-13 molecular forms associated with chronic periodontitis were determined in gingival tissue homogenizates and GCF. A supernatant of gingival fibroblast culture overexpressing MMP-13 was used as the positive control. After boiling for 5 minutes, samples were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V for 1 hour under reductive conditions. Separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane,** the membrane was incubated with signal enhancer†† according to the manufacturer's recommendations, and non-specific interactions were blocked using 1% casein in TBST 0.5% for 1 hour. An anti-human MMP-13 monoclonal antibody‡‡ diluted 1:200 was incubated with membranes overnight at 4°C in the same blocking solution. After washing the membranes with TBST 0.5%, a second anti-mouse

peroxidase conjugated antibody§§ diluted 1:100,000 was incubated for 1 hour, and a positive reaction was identified using the chemiluminiscence method.∥∥ Negative controls were made following the same procedure, without incubating with the primary antibody.

Collection of GCF

After isolating the tooth with a cotton roll, supragingival plaque was removed with curets,¶¶ without touching the marginal gingiva. The crevicular site was dried gently with an air syringe. GCF was collected with paper strips.## Strips were placed into the sulcus/pocket until mild resistance was sensed and left in place for 30 seconds. Strips contaminated by saliva or blood were excluded from the sampled group. Following the GCF collection, the volume of the sample on the paper strips was measured using a calibrated electronic gingival fluid measuring device.*** The readings from the device were converted to an actual volume (µl) by reference to the standard curve. GCF strip samples were obtained from one active site and one inactive site in each patient undergoing periodontitis progression. After GCF collection, strips obtained from the same site were placed in Eppendorf vials and kept under –80°C. GCF was extracted by centrifugation at 12,000 rpm for 5 minutes at 4°C in 50 µl elution buffer containing 50 mM Tris HCl pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$ and 0.01% Triton X-100. The elution procedure was repeated twice,[24] and 21 samples from each site were stored at –80°C until further analysis. MMP-13 activities from GCF samples collected from the active versus inactive sites (21 samples each) were compared in the 21 patients with destructive periodontitis.

MMP-13 Activity Measurements

Aliquots of each GCF sample were assayed by an activity fluorescent assay††† to determine the basal activity of MMP-13, according to the manufacturer's recommendations. Activity was expressed as nanograms of fluorescent product (ng FP) per site and ng FP per microliter of GCF. Additionally, random GCF samples from 10 active and 10 inactive sites were activated with 4-aminophenylmercuric acetate (APMA) to measure total enzyme activity, and the relation between total and basal enzyme activity was obtained to calculate the number of times in which MMP-13 activity was increased with APMA activation.

Data Analysis

The clinical parameters and MMP-13 activity at the site level were expressed as means ± standard deviations. MMP-13 activity values at each site were recorded, and the unpaired Student t test was used to analyze differences in MMP-13 activity levels between active and inactive sites. A statistical significance was considered when $P<0.05$.

RESULTS

Table 1 lists clinical features of the 21 patients studied with progressive chronic periodontitis. All patients had severe/advanced chronic periodontitis. Five males and 16 females were studied, ranging in age from 35 to 62 years (mean age, 45.9 ± 7.7 years). The mean PI was 61.78%, mean BOP was 44.56%, mean PD was 3.63 ± 0.76 mm, and mean attachment level was 4.02 ± 0.52.

Immunohistochemistry of gingival tissue specimens from chronic periodontitis patients revealed that MMP-13 was expressed in gingival tissue from periodontitis, but it was not detected in samples of healthy subjects (Fig. 1). Western immunoblots for MMP-13 showed bands at 60 kDa, corresponding to proenzyme, and stronger bands at 55 and 48 kDa, corresponding to partially and fully activated forms, respectively (Fig. 2). By using *in situ* hybridization techniques, we also found mRNA for MMP-13 in gingival connective tissue samples from patients with chronic periodontitis (Fig. 3). MMP-13 was not detectable in tissue samples or in GCF from healthy patients.

Mean variations of basal activity of MMP-13 expressed as nanograms of fluorescent product per site and per microliter of GCF (FP/site and ng FP/μl GCF, respectively) from active and inactive sites are shown in Table 2. Active sites, identified by an increase in PD during the length of study and associated to periodontal tissue destruction contained higher basal-activity levels of MMP-13 than those found in inactive sites (1.49 ng versus 1.17 ng FP/site and 0.317 ng FP/μl versus 0.249 ng FP/μl, respectively; $P<0.05$). There were increases of 1.94- and 2.51-fold of MMP-13 activity in APMA-activated samples from active sites versus inactive sites, respectively ($P<0.05$), indicating that similar amounts of the enzyme are present in both groups, but a significantly higher proportion of the activated form of MMP-13 is present in active sites.

DISCUSSION

MMP-13 (collagenase-3), a highly expressed collagenolytic MMP in developing bone and cartilage, has been assigned a role in the joint tissue destruction that is a major feature of various forms of human arthritis.[25-27] MMP-13 has critical roles in embryonic development and remodeling of the skeleton in humans. These roles are reflected in the collagenase-mediated destruction of bone and cartilage in several forms of human inflammatory diseases.[28] Our results using immunohistochemistry show that diseased gingival tissues express MMP-13, and the quantity of MMP-13 in the healthy subjects may be less than the minimum detectable level. We demonstrated the presence of MMP-13 by *in situ* hybridization in tissue sections from chronic periodontitis patients and in Western immunoblotting analysis from tissue extracts and GCF. MMP-13 was found only in samples from periodontal disease. The molecular weight of pro-MMP-13 and active MMP-13 is reported to be ~60 and 48 kDa, respectively.[29] Intermediate forms of 55 kDa were also found. Finally, our findings demonstrate that MMP-13 amounts in periodontitis patients are similar, but the level of activation is increased in active sites compared to inactive ones and that basal activity levels are significantly higher in active sites than inactive sites ($P<0.05$). In our previous works, MMP-13 levels were undetectable by "immunodot" blot in all GCF healthy samples (N = 9) and in "immunoWestern" blot samples (data not shown). Thus, it is possible that MMP-13 levels are elevated in periodontitis patients, whereas in healthy subjects the enzyme is undetectable. During periodontitis progression, the main changes between active and inactive sites could occur at the level of pro-MMP-13 activation, determining significant increases of enzyme activity in active sites, thereby resulting in bone and periodontal ligament destruction. MMP-13 is considered a marker of activity progression, and this could be a preliminary approach, which will help to determine the function and

Table 1.

Clinical Characteristics of Periodontitis Patients and Active and Inactive Sites (mean ± SD)

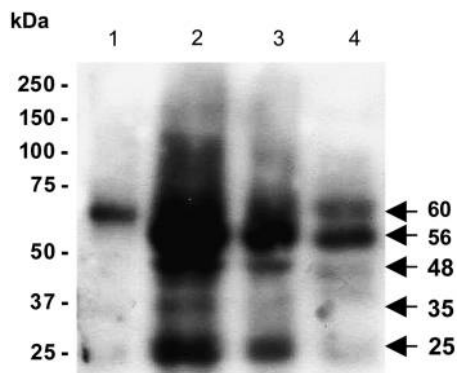| Periodontitis Group (N = 21 patients)		Active Sites (N = 21 samples)	Inactive Sites (N = 21 samples)
Age (years)	45.90 ± 7.70	–	–
Females (%)	76.19	–	–
Mean probing depth (mm)	3.63 ± 0.76	5.89 ± 0.56	5.74 ± 0.60
Mean attachment level (mm)	4.02 ± 0.52	6.59 ± 0.83	6.58 ± 0.92
% sites with plaque	61.78	100	100
% sites with BOP	44.56	100	100
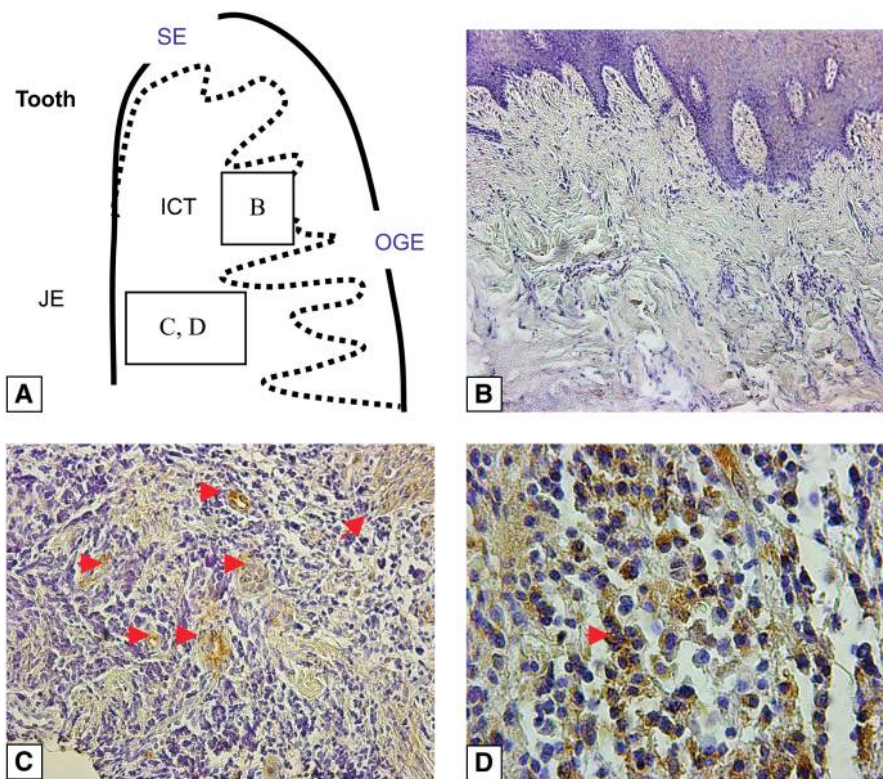
Figure 1.

*MMP-13 immunohistochemistry in gingival tissue from patients with chronic periodontitis. **A)** Diagram illustrating localization of the sections in panels **B through D**. B) Chronic inflammatory cells in a gingival section from healthy control subjects. C) Chronic inflammatory cells in a gingival section from patients with chronic periodontitis. D) Gingival biopsy from patients with chronic periodontitis. Arrowheads indicate immunoreactive cells (Original magnification 40×). (Hematoxyilin and eosin: B and C; anti-human MMP-13 using a DAB chromogen stain and hematoxyilin counterstain: D; original magnification: B, ×4; C, ×20; D, ×40). JE = junctional epithelium; SE = sulcular epithelium; OGE = orogingival epithelium; ICT = infiltrated connective tissue.*
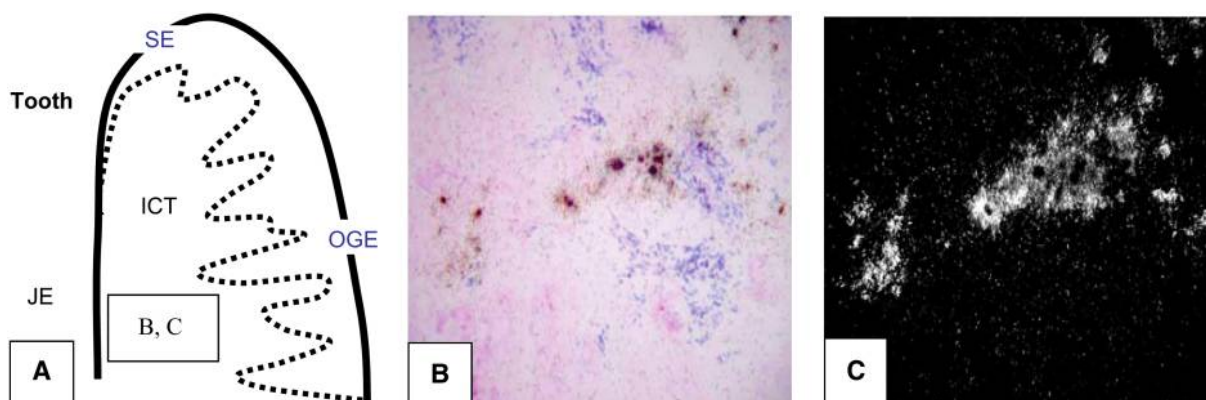

Figure 2.

MMP-13 Western immunoblotting in chronic periodontitis samples: lane 1, positive control: MMP-13 transfected culture fibroblasts supernatant; lanes 2 and 3, gingival biopsies homogenizates; and lane 4, GCF sample. A 60-kDa band corresponding to MMP-13 proform is seen in both positive control and periodontitis samples. Prominent bands of 55-kDa intermediate forms and 48-kDa active forms are seen in lanes 2 through 4 from periodontitis patients.

relevance of this MMP during the progression of the periodontitis.

Disease activity is generally accepted to be a loss of soft or hard tissue attachment to the tooth; a change in probing attachment may represent a true change in the attachment level, a change in tissue tone, or a combination of both.[30] Attention has been focused in the past on the possibility that periodontal disease may not be a continuous process, but may be characterized by episodes of activity followed by periods of relative quiescence.[31] Episodic periodontal probing attachment loss may be associated with variations in the supracrestal inflammatory cell populations where significantly higher numbers of mast cells, monocytes/macrophages, and plasma cells are present in active sites compared to inactive sites.[32] Kiili et al.[33] presented immunohistochemical findings that, in addition to sulcular epithelial cells, found that macrophage-like cells associated with adult periodontitis (chronic periodontitis) lesions can synthesize MMP-13 mRNA and protein.[20] Others reported that MMP-13 is expressed in vitro by stimulated gingival fibroblasts.[34,35]

Our data demonstrate that MMP-13 is present in the GCF of adult patients with progressive chronic periodontitis, and significantly higher activity levels in active sites might correlate the presence of the active form of this enzyme with alveolar bone loss and teeth loss occurring during this disease.[12,36] Our findings demonstrate that MMP-13 could be considered a marker of activity progression. It could regulate osteoblast and osteoclast activity and may be an important target for clinical strategies to modulate the rate of bone formation and resorption produced in chronic periodontitis patients.

Bone volume is maintained through the balance of bone formation by osteoblasts and bone resorption by osteoclasts. The regulation of the balance between osteoblastic and osteoclastic activity is a critical component of normal bone cell biology.[37] Bone resorption involves the removal of mineral and degradation of organic matrix components. Vacuolar type H^+-adenosine triphosphatase (H^+-ATPase) in the plasma membrane of ruffled borders of osteoclasts is involved

Figure 3.
*MMP-13 in situ hybridization demonstration of MMP-13 mRNAs in a gingival biopsy from chronic periodontitis patients. **A)** Diagram illustrating localization of the sections in panels **B** and **C**. B and C) Gingival biopsy from patients with chronic periodontitis showing MMP-13 positive signals. (bright-field image: B; dark-field image: C; original magnification ×40). JE = junctional epithelium; SE = sulcular epithelium; OGE = orogingival epithelium; ICT = infiltrated connective tissue.*

Table 2.

MMP-13 Activity in GCF From Active and Inactive Sites (mean ± SD)

	Basal Activity (ng FP/site)	Basal Activity (ng FP/µl)	GCF (µl)
Active (N = 21)	1.49 ± 0.46*	0.32 ± 0.10†	0.95 ± 0.12
Inactive (N = 21)	1.17 ± 0.20*	0.25 ± 0.04†	0.92 ± 0.09

* MMP-13 basal activity, nanograms of product per site ($P = 0.007$).
† MMP-13 basal activity, nanograms of product per microliter of GCF ($P = 0.008$).

in the demineralization of calcified bone.[38] Additionally, cathepsin K[39] and matrix metalloproteinases, such as MMP-9,[40] could participate in the degradation of organic components in bone matrix, including collagen and non-collagenous proteins, although MMP-9 is also suggested to be indispensable for growth plate vascularization.[41]

MMP-13 is involved in the degradation of extracellular matrix, tumor invasion, and metastasis.[42] Previous studies of MMP-13, using *in situ* hybridization[43,44] and immunohistochemistry,[44] show that osteoblast lineage cells, but not osteoclasts, express and produce MMP-13.[45] Other findings are consistent with those of previous studies of MMP-13 mRNA expression and localization in osteoblast lineage cells.[46] Sakamoto and Sakamaoto[47] proposed that collagenase secreted by osteoblast lineage cells plays a pivotal role in osteoclastic bone resorption. It is generally supposed that collagenase-3 in the vicinity of bone lining cells plays a significant role in exposing mineralized bone matrix by removing non-mineralized collagen fibrils from the bone surface before osteoclastic

bone resorption.[47,48] The removal of these fibrils appears to be essential for differentiation and activation of osteoclasts. It is also possible that MMP-13 under ruffled borders and some clear zones is derived from osteocytes and translocates onto bone surfaces under osteoclasts via the lacunae-canaliculi channel.[46] Thus, the available evidence suggests that collagenase-3 plays a significant role in the initiation and progress of bone resorption. MMP-13 is able to degrade type I, II, and III collagens[29] and aggregan,[49] a cartilage proteoglycan. Moreover, it appears that MMP-13 efficiently degrades type I collagen and may act as an activator of MMP-9.[50] MMP-13 under ruffled borders may also provide a specific microenvironment for the degradation of organic components of bone via the activation of MMP-9.

CONCLUSIONS

To our knowledge, this study provides new and interesting findings on the activity levels of MMP-13 in GCF of sites with episodic loss of connective tissues that implies their potential role in the mechanisms of tissue destruction associated with destructive periodontitis. Additionally, MMPs are known to form tight binding complexes with their natural inhibitors (tissue inhibitors of metalloproteinases [TIMPs]) in extracellular matrix. Further research is needed to clarify the mechanism of MMP-13 activation and involvement of TIMPs.

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