Gelatinolytic activity in gingival crevicular fluid from teeth with periapical lesions

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Objective. Many matrix metalloproteinases (MMPs) have been associated with periodontal tissue destruction during chronic and apical periodontitis. The aim of this study was to determine gelatinolytic activity in gingival crevicular fluid (GCF) from teeth with apical lesions and healthy controls.
Study design. Case-control study.
Materials and methods. After clinical examination, 30 GCF samples were obtained from 20 affected and 20 control teeth. Subjects with periodontal diseases were excluded. Gelatinolytic activity was determined by gelatin zymography and densitometric analysis.
Results. ProMMP-9 levels were significantly increased when compared with controls and were positively correlated with MMP-9 activity in diseased GGF. Similarly, MMP-2 activity, when detected it was exclusively in diseased samples. Additional bands of estimated molecular weights of 60 and 48 kDa were observed.
Conclusions. MMP-9 and −2 are highly increased in GCF from teeth with periapical lesions. These gelatinases could represent useful markers in monitoring chronic apical periodontitis in GCF.

Periapical periodontitis corresponds to inflammation and destruction of periradicular tissues caused by bacterial infection of dental pulp. As a result of bone and apical periodontal extracellular matrix degradation, an apical granuloma or cyst could be formed.[1,2] Several inflammatory mediators like interleukin (IL)-1, IL-6, tumor necrosis factor alpha (TNF-α), prostaglandins, and matrix metalloproteinases (MMPs) have been associated with pulp lesions.[1] The role of these mediators over dissemination of infection and pulpal necrosis is not completely understood, nevertheless, as extracellular matrix degradation by MMPs can be induced by proinflammatory cytokines, it is possible that these enzymes could have an active role in the pathogenesis of pulpal and periapical damage.[3,4]

MMPs are zinc- and calcium-dependent endopeptidases that function at neutral pH. Among them, collagenase activity is critical for fibrillar collagen breakdown, which represents the main constituents of periodontum and alveolar bone. As collagens are cleaved into smaller fragments, they denature at body temperature and are further degraded by gelatinases and other nonspecific tissue proteinases.[5,6] These MMPs have been associated with periodontal tissue destruction in other conditions such as chronic periodontitis and peri-implantitis.[7-9] Recent studies showed that MMP functions are not only limited to matrix degradation, but also bioactive substrates are targeted by MMP proteolytic activity, including inflammatory mediators.[10-13]

Periapical granuloma, like chronic periodontitis, is characterized by alveolar bone destruction as a consequence of bacterial infection and leukocyte infiltration, but initially it is limited to periapical area. Periradicular infiltrates are mainly composed of macrophages, T and B lymphocytes, and plasma cells.[14,15] Macrophages have an important role in matrix turnover regulation and represent the main source of cytokines involved in bone resorption, like IL-1α and IL-1β, and secrete MMPs, including MMP-2 and −9.[4,14,15] The available evidence suggests that MMP-2, −9, and −13 could play a significant role in both the initiation and progress of bone resorption.[16-18] Leonardi et al.[19] proposed that MMPs could be involved in jaw cyst expansion. Immunohistochemical studies have demonstrated MMP expression in periapical lesions,[14,19] but there are no previous studies of MMP activity in gingival crevicular fluid (GCF) of teeth affected with periapical lesions.

GCF contains biochemical mediators that reflect the metabolic status of periodontal tissues,[20] including MMPs. Because of MMPs with gelatinolytic activity could play a role in destruction of periodontium and alveolar bone in periapical lesions, the aim of this study was to determine gelatinolytic activity in GCF from teeth with apical lesions and healthy controls.

MATERIALS AND METHODS

We selected 13 patients consulting at the clinic of diagnosis, School of Dentistry, Universidad Mayor, Santiago, Chile, with one or more periapical lesions detected by periapical radiography due to caries. GCF samples were obtained from around affected teeth and also from healthy equivalent control teeth in each subject, corresponding to the contralateral tooth when possible, or similar tooth from another quadrant. A total of 20 samples and 20 GCF controls were collected. Subjects did not present periodontal diseases or systemic illness and had not received previous antibiotics or nonsteroidal anti-inflammatory drugs during the 6-month period prior to the study. The protocol was clearly explained to all patients and controls, and Institutional Review Board–approved informed consents were signed. Once the patients were diagnosed and samples were taken, they were remitted for endodontic treatment.

Collection of gingival crevicular fluid and elutions

After isolating the tooth with a cotton roll, the crevicular site was then dried gently with an air syringe. GCF was collected with paper strips (ProFlow, Amityville, NY). Strips were placed into the sulcus until mild resistance was sensed and left in place for 30 seconds. Strips contaminated by saliva or blood were excluded. After GCF collection, strips were placed in Eppendorf vials and kept under –20°C. GCF was extracted by centrifugation at 9000g for 6 minutes in 50 μL of elution buffer containing 50 mM Tris HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, and 0.08% Triton X-100. The elution procedure was repeated twice, and samples were stored at –20°C until further analysis.

Gelatin zymography

Gelatin zymography was carried out to assess protease activity. GCF aliquots from diseased (2 μL) and controls (6 μL) were run under nonreducing denaturing conditions, on 10% polyacrylamide gels containing 1 mg/mL gelatin as substrate, soaked twice in 2.5% Triton X-100 for 15 minutes each, and incubated in developing buffer (20 mM Tris, pH 7.4, and 5 mM $CaCl_2$) for 20 hours to develop MMP-2 and −9 activities, and 25 hours for lower molecular weight bands. For visu-

Table I. Clinical parameters in 13 study subjects

Clinical parameter	Mean ± SD	Frequency
Mean age (y)	40.46 ± 15.96	—
Females	—	9/13 (69.23%)
N° Lesions	1.61 ± 0.96	—
Lesion <1cm	—	19/20 (95%)

alization, gels were stained with Coomassie Brilliant Blue R-250 and destained with 10% acetic acid and 20% methanol solution. Gelatinolytic activity was detected as semitransparent bands over a blue background.

Gels were digitized and integrated density was calculated using UN-SCAN-IT gel automated digitizing system V4.1 software (Silk Scientific Corporation, Orem, UT). Results were expressed as arbitrary units of density (pixels) per 30s of GCF collection.

Data analysis

Statistical analysis was done using Systat v.11 software (Systat Software Inc (SSI), San Jose, CA). Wilcoxon-sign test was applied and was considered as significant if P was less than .05. Densitometric intensities were expressed as means ± standard deviation.

RESULTS

In this study we included 13 patients who had one or more teeth affected with periapical lesions in which samples (n = 20) were obtained from diseased and healthy teeth (n = 20) as controls from the same patients, with an average of 1.61 lesions per subject. As shown in Table I, with regard to gender distribution, 31% were men and 69% women with a mean age of 41 years.

GCF analysis (Table II) demonstrated gelatinolytic bands at different molecular weights (Fig. 1). MMP-9 bands (Table II) were detectable in all affected teeth and most controls. Bands corresponding to proMMP-9 were subjected to densitometric analysis and levels of the pro form were higher in the group with periapical lesions ($P < .05$). Similar variations were seen for the active form of MMP-9, which was elevated in disease versus control samples, but this difference was not significant. The proenzyme form predominated over the active enzyme. As shown in Fig. 2, A, MMP-9 activity increased together with proMMP-9 levels, exhibiting a positive correlation (r = 0.5412), whereas this association was not observed in healthy teeth (r = −0.0929), as shown in Fig. 2, B.

Bands corresponding to MMP-2 were not detected in all samples, as observed in Table II. Increased activity and levels were seen in teeth with periapical lesions

Table II. Bands with gelatinolytic activity in GCF from teeth with lesions and controls

	Periapical lesions (n = 20)		Controls (n = 20)		
MMP	*Detectable cases (n)*	*Activity levels (au), means ± SD*	*Detectable cases (n)*	*Activity levels (au), means ± SD*	*p (Wilcoxon sign test)*
proMMP-9	20	44745.780 ± 41116.481	15	16378.291 ± 9881.309	<.05
Active MMP-9	16	17012.658 ± 23807.137	15	7511.967 ± 5428.980	NS
proMMP-2	9	11666.405 ± 10259.011	3	2348.930 ± 1684.589	NS
Active MMP-2	8	3291.133 ± 1755.175	0	—	—
48-56 kDa	8	21738.57 ± 19958.73	5	10223.83 ± 6306.581	NS

GCF, gingival crevicular fluid; MMP, matrix metalloproteinases; NS, not significant.
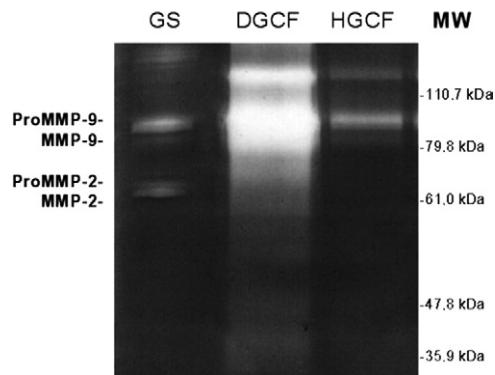

Fig. 1. Gelatinolytic activity in GCF samples from a tooth with periapical lesion and a control. Aliquots of a GCF sample and a contorl were run on 10% polyacrylamide gel containing 1 mg/mL gelatin for gelatin zymogrpahy. Gel was incubated for 20h to develop gelatinase activity. GS: gelatinase standard; DGCF: GCF from diseased tooth; HCGF: GCF from healthy tooth; MW: molecular weight. Bands corresponding to MMP-2 and -9 are observed in diseased sample. Additional bands are seen at 60, 48, and 35 kDA, which could correspond to molecular forms of MMP-13.

compared with controls, but differences were not significant and zymogenic forms predominated over fully active enzyme, as shown in Table II. Active MMP-2, when present, could be found exclusively in disease samples.

Additional gelatinolytic bands that were different from active and pro form of gelatinases were seen in GCF samples (Fig. 1). Thus, high molecular weight bands of 130 kDa were identified. Bands near 56, 48, and 35 kDa were found in many samples. When detected (13/40), 48- to 56-kDa bands were quantified, and gelatinolytic activity was elevated in diseased versus controls, but these differences were not significant (Table II).

DISCUSSION

Apical granuloma or cyst originate from inflammation and destruction of periradicular tissues as a consequence of pulpal necrosis due to bacterial infec-
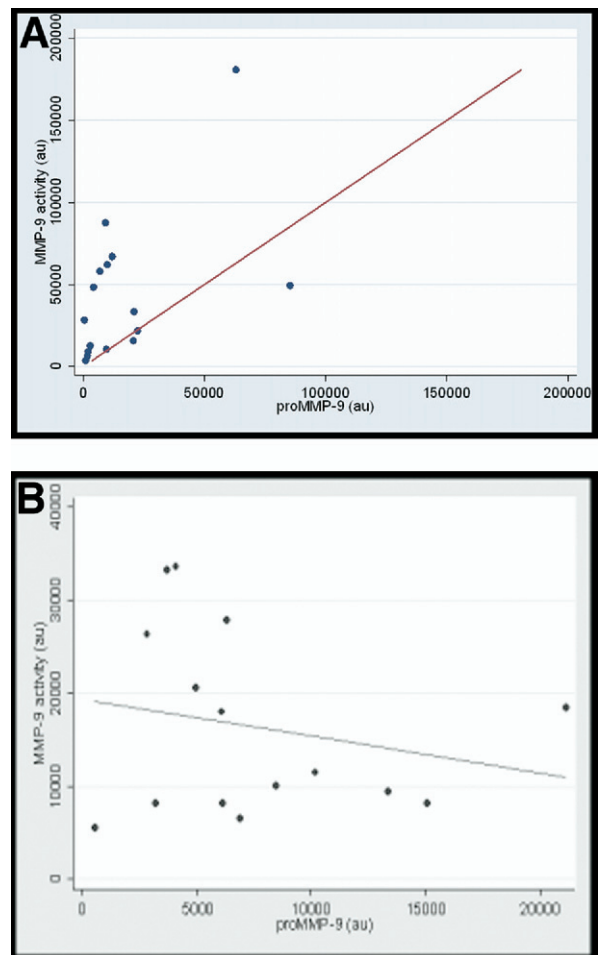

Fig. 2. A, Association between proMMP-9 and MMP-9 activity in GCF from apical lesions. r = 0.5412; p < 0.05. B, Association betwen proMMP-9 and MMP-9 activity in control GCF. R = 0.0929; p > 0.05.

tion[2,15] involving periodontal ligament and alveolar bone loss. Apical and chronic periodontitis may share some pathogenic mechanisms that include inflammatory mediators like IL-1, IL-6, TNF-α, and MMPs.[15]

The results of this study support our hypothesis that MMP-2 and −9 increments in GCF could have an important role in tissue destruction associated with apical periodontitis.

GCF constituents are derived from serum, host cells from gingival tissue, X inflammatory leukocytes and bacteria from subgingival plaque. In physiologic conditions it represents a transudate, but as a result of inflammation and increases in vascular and epithelial permeability, an exudate is formed.[21-23] It carries molecules involved in the destructive process and offers a great potential as a source of factors that may be associated with osteoclastic activity[20] that can be harvested noninvasively from the gingival sulcus or periodontal pocket. Because of the great variability that we have found in GCF volumes and total protein concentration between healthy and inflamed periodontium in a previous study,[24] we agree with Lamster et al.[25] and Golub et al.[26] that the more sensitive method for demonstrating changes in GCF composition is the expression of specific proteins as absolute values under standardized periods of fluid collection.

By gelatin zymography analysis we demonstrated the presence of many gelatinolytic bands in GCF extracted from affected teeth, corresponding mainly to MMP-2 and −9. The more prominent bands corresponded to MMP-9 as described by Makela et al.[27] in periodontal patients and either in diseased or control samples the main forms presented as proenzymes, as described by Korostoff et al.[28] ProMMP-9 levels were significantly increased and were positively correlated with MMP-9 activity in diseased GGF, whereas controls were characterized by lower proenzyme levels and a lack of correlation with its active form, suggesting that apical tissue lost could be closely associated with MMP-9 activity, determined by its activation rate and inhibition by TIMPs (tissue inhibitors of MMPs). Similarly, when MMP-2 activity was detected it was exclusively in diseased samples where it could participate in tissue degradation.

Other bands of high molecular weight were also detected at 130 kDa. They have been described previously as complexes formed by MMP-9 and lipokalin.[6] Macrophages and plasmocytes may express various MMPs, including gelatinases (MMP-2 and −9). The role of these enzymes might be related to the release of growth factors from extracellular matrix to regulate inflammatory reaction or simply to degrade extracellular matrix at the site of inflammation,[4] where collagens type I and III represent the main constituents of periodontal ligament and alveolar bone.[29] In general, collagens can be degraded by the human interstitial collagenases, which include MMP-1, −8, and −13 generating three-fourths to one-fourth length peptides that lose their triple-helical conformation and can be further degraded by gelatinases (MMP-2 and −9).[30]

Occasionally, we observed additional bands of molecular weights similar to those described for MMP-13.[18,31] Bands of 60 kDa have been described as pro MMP-13, whereas 56- and 48-kDa bands could correspond to partially and fully activated forms, respectively.[18,26,32] MMP-13 expression in periapical lesions has been demonstrated by immunohistochemical studies in epithelial cells from proliferating epithelium.[19,33] Uitto et al.[34] proposed that MMP-13 expression in gingiva from chronic periodontitis patients may play a role in growth of epithelium into connective tissue and, similarly, Leonardi et al.[19] associated MMP-13 expression with the migratory state of epithelial cells. Thus, detectable expression of MMP-13 in GCF from periapical lesions could be associated with the presence of epithelial tissue and developing of a radicular cyst from a previous granuloma.

It has been proposed that molecular mechanisms of jaw cyst expansion probably involve interactions of some MMPs and TIMPs.[19] Collagenases (MMP-1 and −8) and gelatinases (MMP-2 and −9) have been identified in jaw cyst wall extracts and cyst fluids.[4] A major pathological event of apical periodontitis is the osteoclastic destruction of bone and dental hard tissues.[2] Previous works have proposed that MMP-2, −9, and −13 play an important role during inflammatory bone resorption and periodontal tissue destruction.[18,35] Under inflammatory conditions, bone resorptive mediators like IL-1 and PGE_2 induce a marked expression of MMPs that include MMP-13, −3, and −2 by osteoblasts that have been associated with increments in bone resorption in mouse calvaria, while MMP-9 is secreted by activated osteoclasts.[17]

Periradicular inflammatory infiltrate is composed mainly of macrophages, T cells, B cells, and occasionally plasmocytes.[14,15] TCD4+ lymphocytes could induce bone resorption by secreting cytokines like RANKL, a key factor in osteoclast differentiation and activation.[15] Macrophages, on the other hand, are important regulators of extracellular matrix turnover and destruction, representing the major source of bone resorption cytokines and by secreting MMPs that include MMP-2, −9, and −13.[15] Previous reports[14] have described significant increases in MMP-2, −3, −8, and −9 in inflamed pulps, produced by both resident and inflammatory cells. Additionally, immunohistochemical-based studies have demonstrated MMP-2 expression in apical granulomas localized to lymphocytes, plasmocytes, and macrophages.[4,14] Thus, MMP-2, −9, and −13 could participate in osteolysis during apical periodontitis. Additionally, MMP-13 has a central role in the activation cascade of other MMPs; MMP-13 can

be activated by other MMPs, including MMP-2, −14, and −3[32]; in turn, it can activate proMMP-9[6,13] and proMMP-13 can be activated itself by active MMP-13.[36] In view of its regulatory effects, it could be understandable that slight changes in MMP-13 activity during apical periodontitis could induce significant destruction of periodontal matrix and changes in immunoinflammatory response.

This is the first study in which GCF is analyzed as a source of molecules released as a consequence of periapical pathosis. Our results support that MMP determinations in GCF could represent a simple and useful tool in monitoring apical inflammation, considering that treatment outcome is difficult to predict based on clinical and radiographic criteria. As long as 4-year follow-up periods are required, whereas misdiagnosis of apical scars and noninflammatory lesions could lead to unnecessary treatments.[37,38] Changes of gelatinolytic activity in GCF from apically comprised teeth could reflect apical inflammation followed by resolution and healing, but longitudinal clinical studies should be conducted.

Similarly, the role of gelatinases and collagenases in pulpal and periapical pathogenesis should be more thoroughly examined. MMP inhibition could provide new treatment modalities in the future.

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