J Periodont Res 2004; 39; 87–92
Printed in the UK. 



JOURNAL OF PERIODONTAL RESEARCH

# *In situ* detection of matrix metalloproteinase-9 (MMP-9) in gingival epithelium in human periodontal disease

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*Smith PC, Muñoz VC, Collados L, Oyarzún AD.* In situ *detection of matrix metalloproteinase-9 (MMP-9) in gingival epithelium in human periodontal disease. J Periodont Res 2004; 39; 87–92. © Blackwell Munksgaard, 2004*

*Background and objective:* As the periodontal lesion develops, the junctional epithelium migrates apically in conjunction with the dissolution of the most coronal Sharpey's fibers. Because matrix metalloproteinase-9 (MMP-9) has been identified in migrating epithelial cells and invading tumors, we propose that this enzyme is produced by gingival keratinocytes in advanced periodontal lesions.

*Methods:* To test this idea, biopsies of inflamed gingival tissues were obtained from patients with advanced periodontitis. Healthy gingival tissue samples were utilized as controls. The presence and activity of MMP-9 was evaluated by combining indirect immunofluorescence of gingival tissue samples and gelatin zymography of gingival epithelium separated from connective tissue.

*Results and conclusions:* The staining pattern showed the presence of MMP-9 in junctional and pocket gingival epithelial cells, polymorphonuclear neutrophils (PMNs) and as a scattered deposit along connective tissues of periodontitis-affected gingival tissues. Gelatin zymography permitted the identification of pro-MMP-9 in surcular/pocket epithelium derived from inflamed gingival tissues. Lower levels of MMP-9 were detected in epithelium not exposed to inflammation. These observations suggest a role for MMP-9 in gingival epithelial response to periodontal infection.

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Key words: gelatin zymography; gingival epithelium; immunohistochemistry; matrix metalloproteinases; periodontal disease

Accepted for publication July 30, 2003

Matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral proteinases capable of degrading extracellular matrix components (1). These enzymes may also process signaling molecules including cytokines and growth factors, modulating their bioactivity (2). Within the MMPs family, the gelatinases form a subgroup of enzymes that includes MMP-2 and MMP-9. These enzymes can degrade basement membrane collagen (3) and other matrix components, such as elastin (4) and collagen type V, VII and X (5–7). Other significant substrates for MMP-9 include interleukin-1 (IL-1) (8), fibrin(ogen) (9) and α1-proteinase inhibitor (10). A critical outcome of periodontal diseases is degradation of the collagenous structure of periodontal tissues and MMPs may play a significant role in this process (11, 12). Polymorphonuclear neutrophils (PMNs) have been recognized as an important source of MMP-9 in human periodontitis (13) and periodontal tissue destruction has been associated with high levels of active MMP-9 in gingival crevicular fluid (14). As the periodontal lesion develops, the junctional epithelium migrates apically leading to loss of attachment. This process requires not only cell proliferation, but also migration of the cells over the tooth and connective tissue substratum that has been modified by the inflammatory process (15). Previous studies have demonstrated that gingival epithelial cells express several MMPs in inflamed periodontal tissues, including MMP-2, -3, -8 and -13 (16–19).

MMP-9 is expressed by gingival keratinocytes *in vitro* (20, 21) and this enzyme is expressed during reepithelialization of palatal wounds (22) and cancer progression (23). Several evidences have suggested that MMPs

function to promote cell migration by clearing extracellular matrix molecules (24). However, a more subtle role has recently been proposed for MMP-9. Studies done with MMP-9 deficient mice showed that this enzyme might inhibit the rate of epithelial wound closure and regulate the inflammatory response (25).

Considering that MMPs expressed by gingival epithelial cells might have a prominent role in cell migration and wound healing, this study was designed to test the hypothesis that MMP-9 is present in gingival epithelium in human periodontal lesions.

## Materials and methods

### Tissue samples

For immunofluorescence of MMP-9 we selected nine adult patients affected by advanced periodontal disease, whose ages ranged between 35 and 65 years. All gingival biopsies were obtained with informed consent from patients attending a private dental practice in Santiago, Chile. The experimental protocol was approved by the Ethical Committee of the Faculty of Dentistry of the University of Chile. None of these patients had received periodontal treatment during the last 12 months. No relevant preexisting medical or drug histories were cited during the last 6 months. Before obtaining a sample, one examiner using a Michigan periodontal probe did a complete periodontal examination in every tooth (six sites per tooth), measuring probing depth, attachment loss, bleeding on probing and presence of plaque. As a condition for inclusion in the study group, periodontal examination should have demonstrated at least three independent periodontal sites with a probing depth of 5 mm or more, 3 mm or more of attachment loss and gingival bleeding upon probing in three independent teeth. Gingival biopsies were obtained during the extraction of hopeless teeth due to advanced periodontitis which had at least one periodontal site with 5 mm of probing depth, 3 mm of attachment loss and bleeding upon probing. All biopsies included both tooth structure and the attached gingival tissues so that the study of junctional epithelial cells would be possible. Healthy control biopsies were obtained from four patients (ages ranging between 12 and 24 years old) who were in need of premolar extraction for orthodontic treatment. None of these patients demonstrated probing depths greater than 5 mm, or attachment loss greater than 3 mm. Healthy gingival sites selected for biopsy demonstrated a probing depth of the sulcus of 3 mm or less, absence of attachment loss and no gingival bleeding upon probing.

For gelatin zymography, a group of six gingival tissue samples were obtained from three patients during tooth extraction due to advanced periodontal disease. Inflamed gingival tissue samples were obtained through an internal bevel incision of the marginal gingiva during tooth extraction. These sites demonstrated at least 5 mm of probing depth, 3 mm of attachment loss and bleeding upon probing. Healthy gingival epithelium was obtained from the palatal mucosa of the same individual through a $2 \times 3$ mm incision of the attached gingiva, located at 4 mm from the gingival margin at the level of the upper first molar.

### Tissue processing

For immunofluorescence, biopsies were immediately fixed in buffered formalin or methacarn for 24 h. After fixation, most of the tooth structure associated with gingival tissues was eliminated using a refrigerated carbide high-speed dental bur. Samples were demineralized in 10% EDTA, pH 7.4 for a period of 20 d. Specimens were dehydrated through graded ethanols, cleared in xylene and embedded in paraffin. Serial sections of approximately 5 μm thickness were collected on poly L-lysine- coated glass slides.

For gelatin zymography, gingival tissues were incubated in a 1 M NaCl solution at 4°C for 96 h (26). After incubation the epithelium was separated from the connective tissue with forceps. Both connective tissue and epithelium were weighted and mixed with the extraction buffer (50 mM Tris-HCl pH 8.0, 1% Triton X100, 150 mM NaCl) in a ratio of 1 : 5 (weight/volume) and homogenized in a glass/glass conical homogenizer for 2 min at 4°C. The homogenate was centrifuged at 13,000 *g* for 15 min at 4°C. The detergent-soluble supernatant was recovered and stored at −70°C until further analysis.

### Immunofluorescence

Sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol, water and phosphate-buffered saline (PBS). In order to expose antigenic sites, a set of sections were treated with 0.1% papain (Sigma P-3250, Sigma, St Louis, MO, USA) in 0.2 M acetate buffer, pH 6.2, for 1 h at 37°C.

For indirect immunofluorescence, sections were preincubated in 5% normal goat serum in 3% PBS-BSA (bovine serum albumin) in a moist chamber for 1 h at room temperature. Sections were incubated with mouse monoclonal antibodies against human MMP-9 (R & D Systems, Minneapolis, MN, USA) at a working dilution of 1 : 100 in PBS containing 1% BSA in a moist chamber at 4°C for 12 h. Sections were washed three times for 5 min each time, and incubated with secondary antibodies conjugated with fluoroscein isothiocyanate (Sigma F-6367) diluted in PBS-BSA at a final dilution of 1 : 500. Cell nuclei were stained with 4 μg/ml Hoechst 33258 diluted in PBS for 30 s. Sections were washed three times in PBS, 5 min each and mounted with glycerol-PBS 1 : 1. Observations were made with a Nikon Microphot-FXA fluorescence microscope (Nikon, Kanagawa, Japan). Images were finally processed by Adobe PhotoShop (Adobe, San Jose, CA, USA). In negative control sections, primary antibodies were replaced by non-immune goat serum diluted in 3% BSA.

### Enzymatic assay by zymography

Detergent extracts of separated gingival epithelium and connective tissue were analyzed by zymography. Zymography was performed using 10% polyacrylamide gels copolymerized with gelatin (1 mg/ml). Proteins loaded in each lane

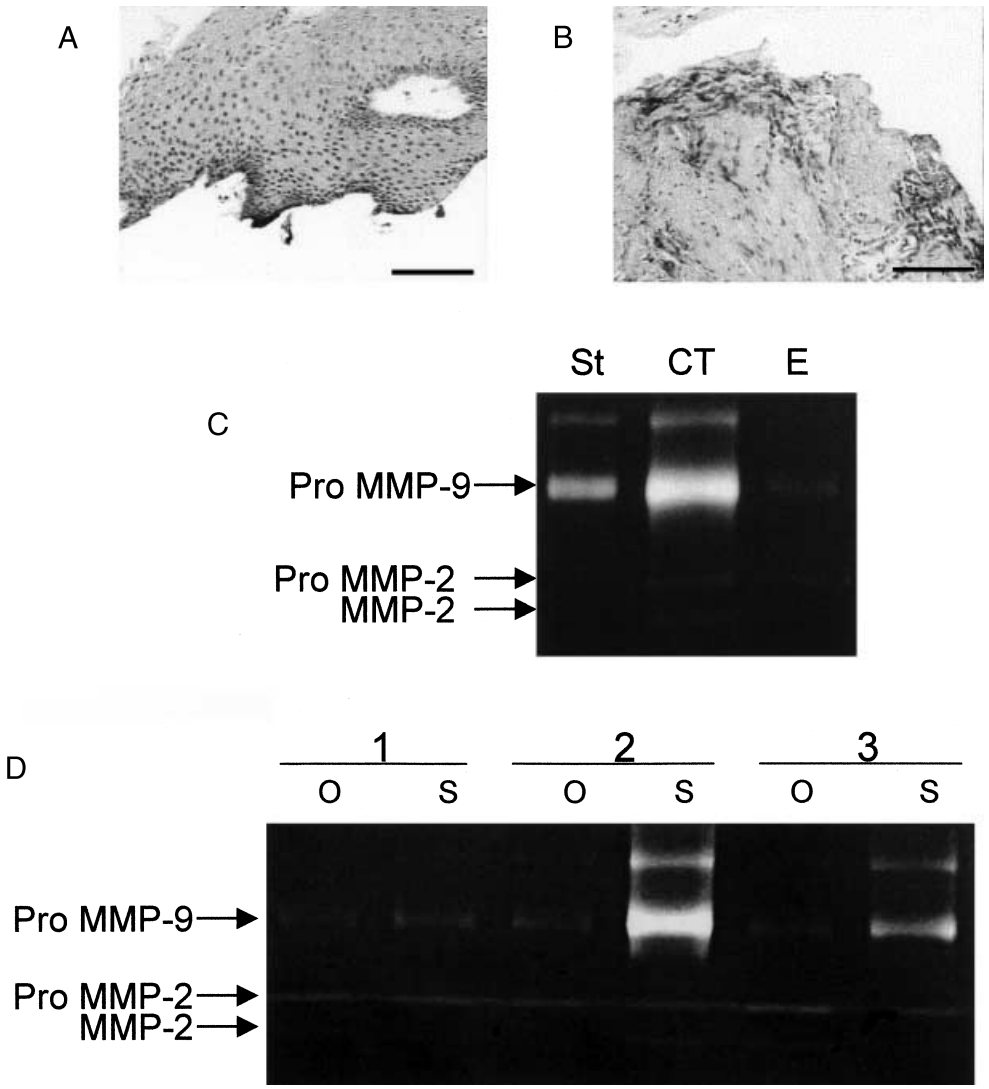


*Fig. 1.* Gelatinolytic activities in healthy and inflamed epithelium. Gingival epithelium (A) and connective tissue (B) were successfully separated after incubation in a 1 M NaCl solution. Bar = 100 μm. C. Tissue homogenates of epithelium and gingival connective tissues demonstrated gelatinolytic activities compatible with the presence of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9). St = standard of purified MMP-9; CT = connective tissue; E = epithelium. D. Gelatinolytic activities of inflamed surcular epithelium (S) and healthy oral epithelium (○) were compared through gelatin zymography in three individuals (1–3). MMP-9 showed a higher gelatinolytic activity in inflamed surcular (S) epithelium when compared to oral epithelium (○).

were normalized by a modified Lowry method (Bio-Rad, Hercules, CA, USA). Samples were diluted in sample buffer (60 mM Tris-HCl pH 6.8 containing 5% sodium dodecyl sulfate, 20% glycerol, 0.03% bromophenol blue) under non-reducing conditions. After electrophoresis, the gel was incubated in 2.5% Triton X-100 for 40 min at room temperature to eliminate the sodium dodecyl sulfate. The gels were then incubated for 24 h in metalloproteinase test buffer (10 mM Tris-HCl pH 8.0, 5 mM $CaCl_2$ at 37°C, 1.25% Triton X-100) at 37°C. Gels were fixed and stained in Coomassie-blue R-250 for 1 h, and destained in an aqueous solution of 10% methanol : 10% acetic acid for 3 h. Gelatinase activity was identified as clear bands against a blue background. Purified 92 kDa gelatinase (Boeringer Mannheim, Mannheim, Germany) and prestained standards (Bio-Rad) were used as molecular weight standards.

## Results

As an initial approach to studying the distribution of active and latent gelatinases in oral tissues, we separated gingival epithelium from connective tissue through incubation in a 1 M NaCl solution. As shown in Figs 1(A and B), this procedure allowed a clear separation of these tissue components as previously demonstrated (26). Gelatin zymography demonstrated that both gingival epithelium and connective tissue expressed gelatinolytic activities corresponding to MMP-2 and -9 (Fig. 1C). Next, we analyzed the gelatinolytic activities in surcular/pocket epithelium obtained from inflamed gingival tissues and in epithelium derived from healthy attached gingiva

of the same individual. Higher levels of pro-MMP-9 were detected in surcular/pocket epithelium when compared to healthy epithelium in two of the three individuals analyzed (Fig. 1D). No remarkable differences were observed in the expression of pro and active MMP-2 between healthy and inflamed epithelial tissues (Fig. 1D).

MMP-9 showed a strong reactivity in periodontitis-affected gingival tissues when visualized by indirect immunofluorescence. MMP-9 expression was observed in all the biopsies obtained from periodontal patients. Two tissue fixatives, methacarn and buffered formalin, were tested in this study. While papain digestion was necessary to identify MMP-9 in buffered formalin-fixed tissues (data not shown), methacarn fixation allowed us to skip antigen retrieval with optimal tissue preservation. In both pocket and junctional epithelium, immunostaining for MMP-9 demonstrated a widespread distribution in most of the cellular layers (Fig. 2A). In pocket epithelium, immunostaining was observed in basal, intermediate and superficial strata (Fig. 2B). In junctional epithelium, basal, suprabasal and directly attached to the tooth cells were also positive for MMP-9 (Fig. 2B). A scattered reactivity for MMP-9 was observed along gingival connective tissue (Figs 2B and C). Strong MMP-9 immunostaining was observed in PMNs transmigrating through gingival blood vessels, infiltrating connective tissue stroma and epithelial intercellular spaces (Fig. 2C). Counterstaining of cell nuclei allowed us to identify epithelial cells as a prominent source of MMP-9 in inflamed gingival epithelium (Fig. 2D). MMP-9 immunoreactivity was mainly detected intracellularly in gingival keratinocytes (Fig. 2D). Healthy gingival tissues demonstrated a low level of immunofluorescence as shown in Fig. 2(E). Omission of primary antibodies in negative control samples showed no immunohistochemical labeling (Fig. 2F).

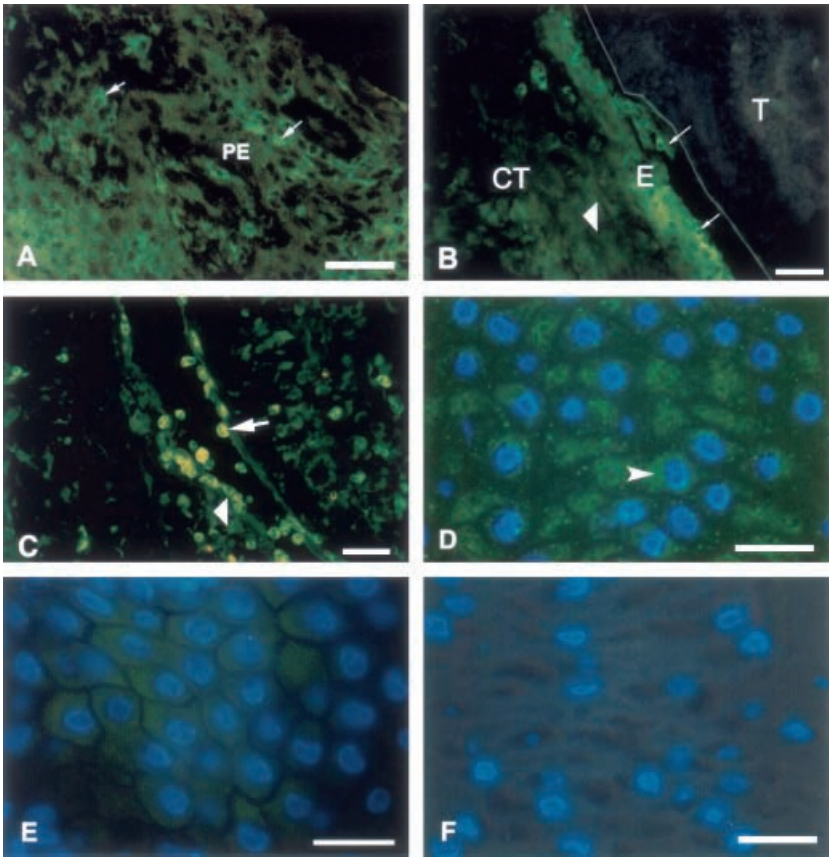


*Fig. 2.* Expression and distribution of matrix metalloproteinase-9 (MMP-9) was studied by immunofluorescence in healthy and periodontitis-affected gingival tissues. A. Inflamed gingival tissue demonstrated MMP-9 positive cells (arrows) within pocket epithelium. Bar = 75 μm. B. Both junctional and pocket epithelium were positive for MMP-9. White line indicates the tooth surface. CT = connective tissue; E = epithelium; T = tooth. Bar = 50 μm. C. Polymorphonuclear neutrophils (PMNs) infiltrating gingival connective tissues were positive for MMP-9. Bar = 50 μm. A scattered staining for MMP-9 was observed throughout gingival connective tissues (arrowhead in B and C) D. Immunostaining for MMP-9 was detected intracellularly in gingival epithelial cells derived from inflamed tissues (arrowhead). Bar = 50 μm. E. Healthy gingival epithelium demonstrated a low level of reactivity for MMP-9. Bar = 50 μm. F. Negative control sample showed no immunofluorescence. Bar = 50 μm.

## Discussion

Several proteolytic enzymes are expressed by gingival epithelial cells in human periodontitis including MMP-2, -3, -8, and -13 (16–19). However, previous studies were unable to identify MMP-9 expression in periodontitis-affected gingival tissues, probably because gingival tissue samples were obtained after initial periodontal therapy (13). The present study permitted the detection of MMP-9 in inflamed gingival tissues obtained from patients affected by advanced periodontal disease. MMP-9 was observed in gingival keratinocytes from junctional and pocket epithelia, and PMNs. By means of gelatin zymography, we observed higher levels of pro-MMP-9 in epithelial tissues exposed to inflammation when compared to healthy oral epithelium. These observations were consistent with MMP-9 immunostaining, which demonstrated a stronger immunolabelling in inflamed gingival epithelium. The pathological samples obtained for the current study originated from hopeless teeth, which were extracted due to advanced periodontitis before initial periodontal therapy. They also demonstrated clear signs of periodontal disease, characterized by the presence of bleeding on probing, probing depth of at least 5 mm and attachment loss of 3 mm or more. Considering the characteristics of our

samples, we believe that the present observations suggest that gingival epithelial cells express MMP-9 during periods of active inflammation.

MMP activity in tissues is regulated at multiple levels, including gene expression, conversion of proenzyme to the activated form, and forming complexes with specific inhibitors (1). Periodontal disease progression is characterized by alternating periods of tissue destruction and remission (27) and periodontal tissue breakdown has been associated with the conversion of proMMP-9 to its activated form in gingival crevicular fluid (14). Gelatin zymography of gingival epithelium demonstrated high proMMP-9 activity under inflammatory conditions. Although our tissue samples probably represented true periodontal lesions, we did not identify if these were in their active/inactive state. Further studies should identify if proMMP-9 may be activated at the gingival epithelium.

During periodontal disease progression, the junctional epithelium migrates apically in conjunction with the dissolution of the most coronal Sharpey's fibers (15). For cellular migration to occur, gingival keratinocytes must detach from the basement membrane, a process that is probably mediated by enzymatic degradation. Active movement of epithelial cells involves adhesion to the extracellular matrix molecules in the contact sites and subsequent contraction of the cytoskeletal elements to bring about the locomotive forces (28). Cell motility also requires release of the cell from the adhesion sites. This is directed, at least in part, by limited proteolysis of the matrix molecules by MMPs (29). Although expression of MMPs by gingival epithelial cells has been associated with cell migration (24), recent studies have suggested that MMP-9 exerts a negative control over the rate of epithelial cell migration and inflammation during wound healing (25). Even though the precise mechanism remains to be established, MMP-9 seems to control the levels of IL-1α in this tissue compartment (25). Aside from these effects, MMP-9 deficient mice demonstrated a deficient remodeling of fibrin(ogen) during wound healing (25). Several studies have provided evidence for degradation of gingival basement membranes exposed to chronic inflammation (30–34). Interestingly, the above-mentioned studies suggest that MMP-9 might exert an opposing role to other MMPs expressed by gingival epithelial cells during periodontal infections.

MMP-9 immunolabelling was observed as a scattered staining distributed throughout gingival connective tissues. MMP-9 is able to bind extracellular matrix molecules such as collagen type I, IV and gelatin and therefore might exert a prolonged biological effect at this tissue level (35). Our observations might reflect the ability of this enzyme to remain bound to extracellular matrix molecules after being released by inflammatory or resident tissue cells.

This study demonstrates the presence of MMP-9 in junctional and pocket epithelium of inflamed gingival tissues and suggests that, in addition to PMNs, keratinocytes might play a role in gingival response to periodontal infection, probably mediated by MMP-9.

## Acknowledgements

We would like to thank Professor Julieta González (Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile) for providing us with the MMP-9 antibody, Mr Juan Méndez for his outstanding technical assistance, and Professor María Antonieta Valenzuela for critical discussions on the article.

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