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Expression analysis of Matrix Metalloproteinase-9 in epithelialized and non-epithelialized apical periodontitis lesions

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Abstract

OBJECTIVE—To determine the expression of matrix metalloproteinase-9 (MMP-9) in apical periodontitis lesions.

STUDY DESIGN—Nineteen epithelialized and eighteen non-epithelialized apical periodontitis lesions were collected after periapical surgery. After histological processing, serial sectioning, H&E staining and microscopic analysis, 10 epithelialized and 10 non-epithelialized lesions were selected for immunohistochemical analysis for MMP-9 and CD 68. At least 1/3 of each specimen was frozen at -70°C for further mRNA isolation and reverse transcription into cDNA for Real-Time-PCR procedures. The relative expression of a target gene was determined in comparison with reference genes (GAPDH, HPRT, β -actin and BCRP).

RESULTS—Polymorphonuclear neutrophils, macrophages and lymphocytes were stained for MMP-9 in both types of lesions, and when present, epithelial cells were also stained. The number and the ratio of MMP-9⁺/total cells were greater in non-epithelialized than epithelialized lesions ($p=0.0001$) and showed a positive correlation to CD68⁺/total cells ($p=0.045$). No significant differences were observed for MMP-9 mRNA expression between epithelialized and non-epithelialized lesions. However, when compared to healthy periapical ligaments, both types of lesions presented increased MMP-9 expression ($p<0.0001$).

CONCLUSION—The present data suggest the participation of several inflammatory cells, mainly CD68⁺ cells, in the MMP-9 expression in apical periodontitis lesions. MMP-9 could be actively enrolled in the ECM degradation in apical periodontitis lesions.

1. INTRODUCTION

Chronic inflammatory diseases of teeth-supporting structures are one of the most significant causes of tooth loss in adults and the most prevalent form of bone pathology in humans and the effects on patients' systemic health has been investigated.¹ During periapical inflammation, host cells in the periapical tissues release many inflammatory mediators, proinflammatory cytokines, and growth factors through innate and adaptive immune responses.²

The MMP family currently consists of 24 members characterized in humans, rodents, and amphibians.³ Initially classified as zinc-dependent proteinases capable of digesting the various structural components of the extracellular matrix (ECM), their specific proteolytic targets have since expanded to many other extracellular proteins. These substrates include an array of other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth factor binding proteins, cell surface receptors, and cell-cell and cell-matrix adhesion molecules.⁴ Regulation of MMP function occurs at multiple levels. MMP mRNA expression is under tight, cell type-dependent control, with expression of individual MMPs associated with specific inflammatory, connective tissue, or epithelial cell types. MMP transcripts are generally expressed at low levels, but these levels rise rapidly when tissues undergo remodeling, such as in inflammation, wound healing, and cancer. MMPs are synthesized as latent enzymes that can be stored in inflammatory cell granules but are more often secreted and found anchored to the cell surface or tethered to other proteins on the cell surface or within the ECM.⁵

Matrix metalloproteinase 9 (MMP-9) or gelatinase B is a multidomain enzyme functioning in acute and chronic inflammatory and neoplastic diseases. MMP-9 is essential for initiating the osteoclastic resorption process by removing the collagenous layer from the bone surface before demineralization can start.⁶

It has been proposed that MMPs play an important role in the destruction of acute inflamed pulp tissue.⁷ MMP-8 together with its fibroblastic counterpart MMP-1 in endogenously active forms is involved in tissue destruction associated with jaw cyst expansion⁸ and MMP-13 could lead to a conversion of a periapical granuloma with epithelium into a radicular cyst.⁹

However, the exact roles of gelatinases in bone destruction associated with apical periodontitis are still unclear. The purpose of this study was to analyze the expression of MMP-9 in the apical periodontitis with or without epithelium.

2. MATERIAL AND METHODS

Thirty seven apical periodontitis lesions were obtained through periapical surgery performed in patients aged 15–55 years (mean age 36.13 years). All patients presented persistence of periapical pathology indicating failure of endodontic retreatment procedures. Patients with contributory medical history, primary or secondary acute periodontitis were excluded from the study. All procedures were realized after obtaining proper IRB approval from the Bauru Dental School, University of São Paulo, and informed consent of the patients.

Tissue Samples and Microscopic Diagnosis

Approximately two-thirds of the tissue of each specimen was immediately immersed in 10% buffered formalin for at least 48 hours (Merck, Darmstadt, Germany) and processed for routine histological examination. Thereafter, the serially sectioned histological specimens were stained with haematoxylin and eosin (HE) for microscopic diagnosis. The tissues collected were constituted by a mass of chronic inflammatory tissue, in which isolated nests of epithelium were sometimes found (19 epithelialized and 18 non-epithelialized apical periodontites). The remaining part of the sample was washed in sterile saline solution and immediately transferred to RNeasy Lysis Buffer (Qiagen, Crawley, UK) and frozen at -70°C for further RNA isolation. The control group was comprised of 8 subjects, (age 17–23, 5 males and 3 females) presenting clinical oral health from whom biopsies of periodontal ligament were taken after tooth extractions previously scheduled for orthodontic reasons.

Immunohistochemistry

The most representative lesions with respect to integrity and tissue arrangement were selected. Twenty apical periodontitis lesions were used (10 without epithelium and 10 presenting epithelium). Sections (3µm thick) were submitted to standard immunohistochemistry procedures (immunoperoxidase method) as described previously.¹⁰ The antibodies used were: rabbit antihuman MMP-9 (C20 sc- goat 6840; Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal antibody or goat polyclonal IgG anti-CD68 (SC-7084; Santa Cruz Biotechnology) and the universal secondary antibody (Universal LSABTM + Kit/HRP – KO690, DaKo, USA). Breast cancer tissue samples were used as positive controls.⁹ Negative controls were obtained by the omission of primary antibodies, which were replaced by 1% PBS-BSA and by (nonimmunized) normal goat serum (X0907; Dako).

Quantitative digital image analysis

The number of cells positively stained for MMP-9 was counted in 10 consecutive microscopic fields (magnification 100X) over total cells counted. The epithelial cell area was separated from the nonepithelial area, for the purpose of cell counting. Digital images were loaded into the Image Pro Plus 4.5 for Windows software (Media Cybernetics, SilverSpring, MD) to count the number of immunostained cells.¹⁰ Statistical analysis was performed using the paired *t* test and a P value of <.05 was considered to be statistically significant.

Real-time polymerase chain reaction (Real-time PCR)

Total RNA and cDNA were prepared as previously published.¹¹ Primer sequences (Table 1) used for gene amplification in Real-Time PCR experiments were designed with the Primer Express 2.0 Software (Applied Biosystems, Foster City, California, USA) connected to the ABI ABI-5700 Thermocycler (Applied Biosystems).

The corresponding Real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E=10^{[-1/\text{slope}]}$.¹²

SYBR Green Dye reagent (Applied Biosystems) was used to quantify the products formed during the Real-Time PCR reaction.¹³ All Real-Time PCR reactions were performed in duplicates. The relative expression of a target gene was determined in comparison with reference genes (GAPDH, HPRT, β-actin and BCRP) through the Ct method.¹⁴ C_T was defined as the point at which the fluorescence rises appreciably above the background fluorescence.

Statistical analysis was performed using GraphPad Prism 4.0 statistical software package for Windows (GraphPad Software, Inc., San Diego, CA). The differences of means between independent samples were calculated using Unpaired sample t-test.

3. RESULTS

3.1 Immunohistochemistry

An immunopositive cytoplasmatic reaction for MMP-9 was observed in all the specimens. Macrophage-like cells were predominantly immunostained for MMP-9 (Fig. 1 A and B, *black arrow*) in both types of lesions. However, some fibroblast-like (Fig. 1 A, *white arrow*), polymorphonuclear neutrophil (PMN)-like (Fig. 1 C and D, *black arrows*) and endothelial-like cells were also stained in both types of apical periodontitis lesions. Thin strands of epithelial cells stained with MMP-9 were also observed (Fig. 1 F). The strong immunostaining of CD68 (a known marker of macrophages) may suggest a predominance of this cell type in the lesions, and in addition, in the view of morphological similarities of MMP-9+ and CD68+ cells in the lesions, suggests that macrophage-like cells could be an important source of MMP-9 in periapical lesions environment (Fig. 1 E). The quantitative analysis of immunostained cells

(Table 2) showed the predominance of the number and ratio of MMP-9⁺/total cells in non-epithelialized rather than in epithelialized lesions ($P < 0.0001$, paired t test). Similar behavior was noted concerning the number and the ratio of CD68⁺/total cells ($P < 0.0001$; unpaired t test with Welch correction). There was a positive correlation (Fig. 2) between the number and ratio of MMP-9⁺/total cells and CD68⁺/total cells in non-epithelialized lesions ($P = 0.045$, $r^2 = 0.4121$, slope = 0.5433 ± 0.2294). No significant correlation was found to epithelialized lesions (not shown). CD68⁺ cells were observed mainly in the central areas in both lesion types. Negative controls obtained by replacing primary antibodies with PBS/BSA showed no stained cells (data not shown).

3.2 Real time PCR

No significant differences were observed for MMP-9 mRNA expression between epithelialized and non-epithelialized lesions. However, when compared to healthy periapical ligaments, both types of lesions presented increased MMP-9 expression ($p < 0.0001$, One-Way ANOVA, followed by Bonferroni's post-test, Fig. 3).

4. DISCUSSION

Periapical lesions are the consequence of an immune-inflammatory reaction raised against infectious agents present in the periapical environment. Epithelial cells with latent capacity to proliferate, derived from epithelial cell rests of Malassez are a common finding in this kind of periapical pathology.¹⁵

Apical periodontitis can be classified as cysts or granulomas. Also, when the differentiation between "real" cysts and epithelialized granulomas is not possible/effective/clear they could be classified as epithelialized or non-epithelialized. Despite such differences, they share common characteristics, such as the maintenance of a chronic inflammatory infiltrate, usually associated with an increased rate of ECM degradation and bone resorption activity.¹⁶

In this study, the tissues removed surgically from the apical region were diagnosed as periapical periodontitis lesions and classified as epithelialized or non-epithelialized and were evaluated regarding the expression of MMP-9 and CD68. Both types of lesions, demonstrated immunostaining for MMP-9. Other MMPs than MMP-9 have been observed in human and experimental periapical lesions.^{2,9,17} In addition, MMP-9 was found to be produced by human pulp and periodontal ligament cells stimulated with endodontic pathogens.¹⁸ Molecules of the ECM can be broken down by MMPs, which are secreted locally by the surrounding cells. Such degradation of the ECM is essential for cell migration and in the release and activation of growth factors.¹⁹ Increased MMP-9 expression/activity has been linked with tissue destruction, and is specially found to be upregulated in bone resorption sites, such as in rheumatoid arthritis and in periodontal lesions.^{20,21}

The present study showed that MMP-9⁺ stained cells were more frequently observed in non-epithelialized lesions when compared to epithelialized lesions ($p = 0.0001$). In addition, the number of cells positive to CD68, were also found to be increased in non-epithelialized lesions. In agreement, a previous study demonstrated increased number of several leukocyte subsets, including CD68⁺ cells, in non-epithelialized lesions versus epithelialized periapical lesions.²² Macrophages play an important role in regulating the turnover of extracellular matrix in both normal and pathologic conditions by the secretion of proteases, including MMPs, protease inhibitors, cytokines and chemokines.²³ MMP-9, in particular, is involved in macrophage recruitment and tissue remodeling that allows the formation of a tight and well-organized granuloma.²⁴ In fact, a positive correlation was found between the number of MMP-9 and CD-68 positive cells, suggesting that macrophages could be an important source of MMP-9, and/or that MMP-9 could play an important role in the migration of this leukocyte subset to

periapical lesions. Also, cytokines and chemokines derived from macrophages may be implicated in the proliferation of Malassez epithelial rests from which cyst lining originates^{22,25} and therefore the differential CD68 counts could be related with the granuloma to cyst transition. Similarly, it has been demonstrated that MMP-13 is highly upregulated when an epithelium is present in periapical pathologies, which could lead to the conversion of an epithelialized periapical granuloma into a radicular cyst.⁹ Once the molecular mechanisms of the putative conversion of apical granulomas into cysts are still poorly understood, further studies are required to solve this question.

Despite the differences observed between the number of MMP-9+ cells in both types of lesions, no significant differences were found when comparing MMP-9 mRNA expression in epithelialized and non-epithelialized apical periodontitis lesions. Considering that mRNA was extracted from homogenized tissues including many different cell types, small sample size and/or large individual variation, a difference in mRNA expression between the two groups could not be discarded. However, in both types of lesions the expression of MMP-9 was found to be significantly higher than in normal periodontal ligament samples, suggesting that MMP-9 could play a role in the tissue destruction associated with periapical lesions development/progression. In fact, MMPs inhibition has been demonstrated to inhibit bone loss in several experimental models²⁶ and also in humans.^{27,28,29}

Taken together, our results suggest that MMP-9 is actively enrolled in the degradation of the apical extracellular matrix. Nonetheless, additional investigations should be further pursued to better clarify the role of MMP-9 and other proteinases in these lesion types.

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Figure 1.
The anti-MMP-9 stained macrophages-like cells (A, B - *arrow*), fibroblastlike cells (C, - *black arrow*), PMN-like cells (D - *white arrow*), and epithelial cells (D, E - *black arrow*). CD68 positive cells stained (F - *black arrow*). Original magnifications: 40X (A, D) and 100X (B, C, E, F).

Figure 2.
Pearson Correlation plot of CD-68 versus MMP-9 immunopositive cells in non-epithealized
($P=0.045$, $r^2=0.4121$, slope= 0.5433 ± 0.2294).

Figure 3.

Relative mRNA expression of MMP-9 in human apical periodontitis lesions and healthy periapical ligaments. Relative expression was analyzed by Real-time PCR ($p > 0.05$, Unpaired t test) and different letters (a, b) indicates $p < 0.05$. The values represent gene output after normalization relative to GAPDH, HPRT, β -actina and BCRP.

Table I

Sequence of primers used in this study for the amplification of the respective genes

Gene	Primer sequence
human <i>MMP-9</i>	F: 5' CCTGGAGACCTGAGAACCAATC 3' R: 5' GATTTCGACTCTCCACGCATCT3'
human <i>βACTIN</i>	F: 5' GGCACCCAGCACAAATGAAG 3' R: 5' CCGATCCACACGGAGTACTTG 3'
human <i>BCRP</i>	F: 5' CCTTCGACGTCAATAACAAGGAT 3' R: 5' CCTGCGATGGCGTTCAC 3'
human <i>GAPDH</i>	F: 5' ACCCACTCCTCCACCTTTGA 3' R: 5' CTGTTGCTGTAGCCAAATTCGT 3'
human <i>HPRT</i>	F: 5' GAACGTCTTGCTCGAGATGTGA 3' R: 5' TCCAGCAGGTCAGCAAAGAAT 3'

Table II

Distribution of immunostained cells for MMP-9 according to each lesion type

<i>Marker</i>	<i>Lesions</i>	
	<i>Non-epithelialized</i>	<i>Epithelialized</i>
	<i>Mean ± SD</i> <i>(ratio ± SD)^a</i>	<i>Mean ± SD</i> <i>(ratio ± SD)^a</i>
<i>MMP-9</i>	36.30 ± 17.72 (0.421 ± 0.129)	30.61 ± 13.23 (0.309 ± 0.102)
<i>CD68+</i>	28.54 ± 7.23 (0.381 ± 0.040)	24.05 ± 7.59 (0.307 ± 0.068)

^aThe values inside the parentheses represent the ratio of the number of immunostained cells over the total number of cells in the field and standard deviation (n=100).