DNA Methylation of *MMP9* Is Associated with High Levels of MMP-9 Messenger RNA in Periapical Inflammatory Lesions

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Abstract

Introduction: Matrix metalloproteinases (MMPs) are the major class of enzymes responsible for degradation of extracellular matrix components and participate in the pathogenesis of periapical inflammatory lesions. MMP expression may be regulated by DNA methylation. The purpose of the present investigation was to analyze the expression of *MMP2* and *MMP9* in periapical granulomas and radicular cysts and to test the hypothesis that, in these lesions, their transcription may be modulated by DNA methylation. **Methods:** Methylation-specific polymerase chain reaction was used to evaluate the DNA methylation pattern of the *MMP2* gene in 13 fresh periapical granuloma samples and 10 fresh radicular cyst samples. Restriction enzyme digestion was used to assess methylation of the *MMP9* gene in 12 fresh periapical granuloma samples and 10 fresh radicular cyst samples. *MMP2* and *MMP9* messenger RNA transcript levels were measured by quantitative real-time polymerase chain reaction. **Results:** All periapical lesions and healthy mucosa samples showed partial methylation of the *MMP2* gene; however, periapical granulomas showed higher *MMP2* mRNA expression levels than healthy mucosa (P = .014). A higher unmethylated profile of the *MMP9* gene was found in periapical granulomas and radicular cysts compared with healthy mucosa. In addition, higher *MMP9* mRNA expression was observed in the periapical lesions compared with healthy tissues. **Conclusions:** The present study suggests that the unmethylated status of the *MMP9* gene in periapical lesions may explain the observed up-regulation of messenger RNA transcription in these lesions. *(J Endod 2016;42:127–130)*

Key Words

Matrix metalloproteinase, messenger RNA, methylation, periapical lesion, radicular cyst, transcription

The development of periradicular bone pathologic alterations, such as periapical granulomas or radicular cysts, results from the infection by microorganisms in the root canal system (1). These lesions can show concomitant resorption of hard tissues and the destruction of the periradicular periodontal ligament (2). Radicular cysts develop from the proliferation of dormant epithelial rests of Malassez (3) under the influence of growth factors (4).

Matrix metalloproteinases (MMPs) are an important family of metal-dependent endopeptidases and represent the major class of enzymes responsible for degradation of extracellular matrix components (5). Expression of most MMPs is normally low in tissues and is induced when remodeling of ECM is required, such as in inflammation, wound healing, and cancer (6). Therefore, excessive secretion of these enzymes is the hallmark of apical periodontitis, suggesting the direct participation of MMPs in tissue remodeling/destruction during lesion development (7–9).

Accumulating data from various studies strongly suggest that MMPs are primarily regulated at the transcriptional level (10–12) although the factors governing the expression of specific MMPs in different cells and tissues have yet to be fully defined. This intricate transcriptional regulation of MMPs becomes even more complicated when taking into account the impact of promoter polymorphisms, epigenetic regulation mechanisms (11), and post-transcriptional processes (13).

For instance, previous studies have suggested that polymorphisms in candidate genes for periapical disease (ie, MMPs and interleukins) may predispose individuals to disease development and/or progression, possibly through alterations in gene transcription (8, 14). DNA methylation is another regulatory mechanism and results in the transcriptional inactivation of the gene. Increased methylation/hypermethylation in the promoter region of a gene leads to decreased transcription, whereas hypomethylation results in transcriptional up-regulation (15). We have previously shown the involvement of DNA methylation as a regulatory mechanism of cytokine genes in periapical lesions (16, 17). Partially methylated periapical lesion samples showed increased interferon-gamma (*IFNG*) messenger RNA (mRNA) expression levels compared with totally methylated samples (16). Similarly, periapical lesion samples showing high forkhead box P3 (*FOXP3*) gene methylation levels presented reduced *FOXP3* mRNA expression (17).

Because MMPs play an important role in periapical lesion development (15–17) and up-regulated MMP-2 and MMP-9 expression in particular has been consistently reported in periapical disease, (7, 8) the purpose of this study was to associate the DNA

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methylation profile of *MMP2* and *MMP9* genes in periapical granulomas and radicular cysts with mRNA expression levels.

Methods

Sample Collection

This study was approved by the Institutional Review Board at Universidade Federal de Minas Gerais (CAAE -0462.0.203.000-11), Belo Horizonte, Brazil, and all patients signed an informed consent form.

Twenty-three fresh tissue samples were included in the study. Samples were obtained from male and female patients between the ages of 16 and 75 years old referred for dental extraction at the oral surgery clinic and who presented with radiographic evidence of periapical alveolar bone loss around a tooth without pulpal sensitivity and without endodontic treatment. Patients who had taken systemic antibiotic and/or anti-inflammatory drugs for 2 months before the surgery were not included in the study. Upon collection, each sample was immediately sectioned into 3 equal fragments as follows:

1. One fragment was stored in Tissue-tek (Sakura Finetek Inc, Torrance, CA) at −80°C for posterior DNA extraction.
2. The second fragment was stored in RNA Holder (BioAgency Biotecnologia, São Paulo, Brazil) at −80°C for posterior RNA extraction.
3. The third fragment was stored in 10% formalin solution for posterior histopathological processing and analysis (data not shown).

Lesions were diagnosed as periapical granulomas in the absence of an odontogenic epithelium, whereas lesions classified as radicular cysts exhibited a cystic cavity lined by an odontogenic epithelium (18). The periapical granuloma group was composed of 13 lesions (7 from male patients and 6 from females). The radicular cyst group was composed of 10 lesions (6 from males and 4 from females). Twelve fragments of healthy mucosa samples with no clinical evidence of inflammation, collected during impacted third molar extractions, were used as normal tissue controls.

DNA Isolation and Methylation Analysis of *MMP2* and *MMP9*

Genomic DNA was isolated from the tissue samples using a Qiagen DNeasy Tissue Kit (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. To assess the *MMP2* gene CpG island methylation, genomic DNA was modified by sodium bisulfite treatment as described elsewhere (19) and subsequently submitted to methylation-specific polymerase chain reaction (MSP-PCR) (20) by using previously described primer pairs designed to specifically recognize methylated (F: 5′-GCGGTTATACGTATCGAGTTAGC-3′ and R: 5′-ACTCTTTATCCGTTTTAAAAACGAC-3′; amplicon 205 bp) and unmethylated DNA sequences (F: 5′-GGTGGTTATATGTATTGAGTTAGTGA-3′ and R: 5′-ACTCTTTATCCATTTTAAAAACAAC-3′, amplicon 206 bp) (21). PCR products were subjected to electrophoresis in 6.5% polyacrylamide gels and silver stained. The samples were considered as nonmethylated when only the reactions with the unmethylated primers showed amplification, partially methylated when both PCR reactions amplified (with methylated and unmethylated primers), and completely methylated when a positive amplification was obtained only with the methylated primer pair (22). Bisulfite-treated unmethylated DNA of peripheral blood mononuclear cells was used as the unmethylated reaction positive control, and the methylation-induced DNA of the same cells treated by the MSssI methylase enzyme (New England Biolabs, Beverly, MA) and bisulfite was used as a positive control of the methylated reaction, which is similar to a previous study (23).

Methylation of the promoter region of *MMP9* was assessed using restriction enzyme digestion. The methylation-sensitive restriction enzyme AciI (New England Biolabs) was used to assess the methylation of CG dinucleotides in sites located at positions −185, −223, and −233 of the *MMP9* promoter according to a bioinformatics web site (http://www.restrictionmapper.org). Restriction enzymes cut DNA at unmethylated CG dinucleotides, but they do not cleave methylated cytosines. Two hundred nanograms of genomic DNA were digested with the restriction enzyme AciI according to the manufacturer's protocol. Digestion was followed by PCR amplification using previously described primer pairs (F: 5′-GCTTCATCCCCCTCCCTCC-3′, R: 5′-AGCACCAGGACCAGGGGC-3′; amplicon 369 bp) (21). PCR products were subjected to electrophoresis in 6.5% polyacrylamide gels and silver stained. The detection of a PCR band represents a methylated profile of the *MMP9* gene, whereas the failure to detect a PCR band represents an unmethylated profile caused by DNA cleavage by the restriction enzyme. In each PCR reaction, undigested DNA of each sample was also performed as a positive control. Undigested and digested PCR products were subjected to electrophoresis side by side. Unmethylated DNA of peripheral blood mononuclear cells was used as an unmethylated positive control.

MMP2 and *MMP9* Gene Expression

Trizol reagent was used for total RNA extraction from tissue samples following the manufacturer's instructions (Invitrogen, Carlsbad, CA) and treated with DNAse (Invitrogen Life Technologies). RNA integrity was analyzed by using 1% agarose gel electrophoresis. Reverse transcription of 1 μg RNA to complementary DNA (cDNA) was performed using SuperScript III First-Strand (Invitrogen) according to the manufacturer's recommendations. Beta-actin cDNA (amplicon 353 bp) was amplified as a control for cDNA quality. All reactions were run in a StepOne Real time PCR System using the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK). *MMP2* and *MMP9* mRNA expression was assessed using the following primer sequences: for *MMP2*, forward: 5′-AGCTCCCGGAAAAGATTGATG-3′ and reverse: 5′-CAGGGTGCTGGCTGAGTAGAT-3′ (101-bp amplicon) and for *MMP9*, forward: 5′-GAGGTTCGACGTGAAGGCGCAGAT-3′ and reverse: 5′-CATAGGTCACGTAGCCCACT TGGT3′ (200-bp amplicon) (21). Relative gene expression was calculated by using the $2^{-\Delta\Delta ct}$ method (24) and normalized to the expression of endogenous beta-actin. We used placenta tissue samples as calibrator for both *MMP2* and *MMP9* expression. All reactions were run in duplicate.

Statistical Analysis

The Fisher exact test was used to evaluate the association between variables for categoric data. The Shapiro-Wilk test was performed to evaluate the distribution of all quantitative variables. The nonparametric Mann-Whitney test was used to compare 2 groups of cases on 1 variable. Statistical analyses were performed by using SPSS 17.0 software (SPSS Inc, Chicago, IL); P values < .05 indicate a statistically significant difference.

Results

Because all periapical granuloma (n = 13), radicular cyst (n = 10), and healthy mucosa (n = 12) samples were partially methylated for the *MMP2* gene, comparison of the methylation status of the gene between the groups was not feasible. However, despite the partial *MMP2* methylation findings, periapical granulomas showed higher *MMP2* mRNA expression levels than healthy mucosa (P = .014) (Fig. 1). No statistical difference was found in *MMP2* mRNA expression

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