High Matrix Metalloproteinase Activity Is a Hallmark of Periapical Granulomas

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Abstract

Introduction: The inability to distinguish periapical cysts from granulomas before performing root canal treatment leads to uncertainty in treatment outcomes because cysts have lower healing rates. Searching for differential expression of molecules within cysts or granulomas could provide information with regard to the identity of the lesion or suggest mechanistic differences that may form the basis for future therapeutic intervention. Thus, we investigated whether granulomas and cysts exhibit differential expression of extracellular matrix (ECM) molecules. **Methods:** Human periapical granulomas, periapical cysts, and healthy periodontal ligament tissues were used to investigate the differential expression of ECM molecules by microarray analysis. Because matrix metalloproteinases (MMP) showed the highest differential expression in the microarray analysis, MMPs were further examined by *in situ* zymography and immunohistochemistry. Data were analyzed by using one-way analysis of variance followed by the Tukey test. **Results:** We observed that cysts and granulomas differentially expressed several ECM molecules, especially those from the MMP family. Compared with cysts, granulomas exhibited higher MMP enzymatic activity in areas stained for MMP-9. These areas were composed of polymorphonuclear cells (PMNs) in contrast to cysts. Similarly, MMP-13 was expressed by a greater number of cells in granulomas compared with cysts. **Conclusion:** Our findings indicate that high enzymatic MMP activity in PMNs together with MMP-9 and MMP-13 stained cells could be a molecular signature of granulomas unlike periapical cysts. *(J Endod 2009;35:1234–1242)*

Key Words

Extracellular matrix componenets, matrix metalloproteinases, periapical cyst, periapical granuloma, periodontal ligament

Periapical disease represents the progression of a bacterial infection from the dental pulp to the apical foramen that results in a localized inflammatory response concomitant with bone resorption (1–4). Periapical lesions include granulomas and cysts, and both are thought to represent different stages of the same inflammatory process (5–7).

Differential diagnosis of granulomas and cysts using radiographic analysis is problematic. Although preliminary studies have proposed that computed tomography scans and ultrasound with power Doppler flowmetry can provide an additional diagnostic tool in endodontics (8–10), it is widely accepted that histologic evaluation is necessary to confirm diagnosis (4, 11–14). However, microscopic examination can only be performed after the periapical disease is removed, which is a limitation because in most cases root canal treatment is performed without removing the lesion. This inability to identify the status of periapical disease makes treatment outcomes unpredictable because cysts exhibit lower healing rates and generally require additional surgical treatment (6, 15–18).

Identifying extracellular matrix molecules (ECMs) specific to human periapical cysts or granulomas can provide information to potentially discriminate between these lesions. Specific proteins present in the extracellular matrix and their respective receptors may offer the basis to develop novel approaches aiming to detect disease biomarkers and therefore to improve diagnosis for cysts and granulomas before performing root canal treatment. Possible likely candidates as biomarkers for periapical inflammation include proteases that are responsible for ECM degradation such as matrix metalloproteinases (MMPs). MMPs are a family of metal-dependent endopeptidases, which are secreted as inactive proenzymes (zymogens) and activated in tissues by cleavage of the propeptide (19–23). Although MMPs have been reported in periapical lesions (11, 24–30), a direct comparison of MMPs in cysts and granulomas has not been undertaken. Because MMPs must be activated to exert their function, it is also important to localize areas of proteinase activity within lesions. Thus, the aim of this study was to compare the expression of several ECM molecules and cell membrane receptors within different cellular components from human periapical granulomas and cysts. In addition, we sought to examine the presence and activity of MMPs in these tissues because of their reported involvement in these lesions and because our initial data revealed differential expression of this class of proteins in these tissues.

Material and Methods

Collection of Samples

Formalin-fixed paraffin-embedded sections from 10 periapical granulomas and 10 periapical cysts were obtained from the archives of the Oral Pathology Biopsy Service at the University of Michigan School of Dentistry after institutional review board

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Supported by Grant NIH-R01-DE013725 to YLK and a CAPES Foundation Fellowship to FWGPS (0668/07-9).

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0099-2399/$0 - see front matter

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doi:10.1016/j.joen.2009.06.008

approval. Periodontal ligament (PDL) tissues obtained from the middle third of the dental root of extracted healthy teeth, after institutional review board approval, were used as controls.

Histological Examination of the Samples

Histological examination was performed by a pathologist on hematoxylin-eosin–stained slides of tissues from the apical regions of nonvital teeth. Periapical cysts were selected based on the presence of granulation tissue with lining stratified squamous epithelium. Periapical granulomas were selected based on the presence of granulation tissue without lining epithelium (16, 17, 31). Fibroblast-like cells were characterized by their spindle-shaped morphology, mononuclear inflammatory cells were identified as cells with a large single nucleus, and PMNs were identified by their multilobed nuclei.

RNA Extraction and Microarray Analyses

Paraffin-embedded tissue sections were deparaffinized, exposed to protease digestion, and immersed in Trizol Reagent (Invitrogen Corporation, Carlsbad, CA) for RNA extraction. Extracted RNA was purified by using the RNEasy Micro Kit (Qiagen, Valencia, CA). The quality and concentration of isolated RNA was evaluated by using a capillary electrophoresis system in an RNA 6000 Pico LabChip (Agilent 2100 BioAnalyzer; Agilent Technologies Inc, Santa Clara, CA). Analysis of expression of ECM molecules (Table 1) in tissues was performed using focused complementary DNA (cDNA) microarrays (SuperArray; Bioscience Corporation, Frederick, MD). Ten micrograms of total RNA were used as a template to generate biotin-16-2′-deoxyuridine-5′-triphosphate, tetralithium salt (biotin-16-dUTP) labeled cDNA probes. cDNA probes were denatured and hybridized at 60°C with the SuperArray membranes, which were developed using chemiluminescence. Gene expression was evaluated by densitometric analysis of the membrane spots. Comparison among groups was performed on a gene-by-gene basis after normalization by β-actin messenger RNA expression.

In Situ Zymography

Five-micrometer-thick tissue sections were immersed in sodium borohydride (1 mg/mL) followed by incubation with a fluorescein isothiocyanate (FITC)-bound gelatin substrate (DQ Gelatin; Molecular Probes, Eugene, OR) dissolved in agarose (0.1 mg/mL) for 3 hours at 37°C in a humidified light-protected chamber. Nuclei were counterstained by adding 4'-6-diamidino-2-phenylindole (DAPI, 0.5 μg/mL) to the incubation medium. Control slides were preincubated in 20 mmol/L ethylene diamine tetraacetic acid (EDTA; Sigma, St Louis, MO) for 1 hour, and then EDTA was added to the incubation medium. The quantification of gelatinolytic activity in the sections was assessed by counting the number of spots of fluorescence in representative areas (40× magnification) and expressed as number of spots of enzymatic activity per millimeters squared.

Immunohistochemistry

Specific MMPs were detected by immunohistochemistry. Tissue sections were quenched in a 6% H_2O_2 methanol solution for 30 minutes and boiled in 10 mmol/L sodium citrate (pH 6.0) at 93°C for 15 minutes for antigen retrieval. Nonspecific binding was blocked with 1% bovine serum albumin (Sigma) for 30 minutes, and sections were incubated for 1 hour with primary antibodies for MMP-2 (5 μg/mL, MAB3308; Chemicon, Temecula, CA), MMP-9 (5 μg/mL, MAB3309, Chemicon), and MMP-13 (5 μg/mL, M4052, Sigma). Sections were incubated with secondary antibody followed by streptavidin horseradish peroxidase and 3,3'-diaminobenzidine (DAB500 Chromogen System, Biocare Medical). Tissues were counterstained with Mayer's hematoxylin and mounted using standard protocols. Negative controls consisted of replacing the primary antibody with mouse or rabbit immunoglobulin G. The number of positive cells was calculated for each antibody in three representative fields of view (100× magnification).

Statistical Analysis

Data were analyzed by using one-way analysis of variance followed by the Tukey test ($\alpha = 0.05$).

Results

Periapical Granulomas Exhibit a Higher Percentage of PMNs Compared to Cysts

Mononuclear inflammatory cells were the most prevalent cells found in both cysts and granulomas (59.8% ± 19.8% vs 43.1% ± 3.8%, respectively; $p > 0.05$). Similar quantities of fibroblast-like cells were also observed in both lesions (24.1% ± 8.4% in cysts vs 24.6% ± 4.0% in granulomas; $p > 0.05$). In contrast, PMNs were more prevalent in periapical granulomas compared with cysts (32.1% ± 5.8% vs 15.9% ± 7.5%, respectively; $p < 0.05$). Inflammatory activity, assessed as a ratio of inflammatory cellular components (PMNs plus mononuclear cells) to fibroblast-like cell count, was similar for both cysts and granulomas (3.2 and 3.3, respectively); however, the composition of inflammatory activity was different between the lesions because PMNs predominated in granulomas. Periapical cysts were surrounded by a stratified squamous epithelium, and fibroblast-like cells were observed in the stroma and surrounding the lesions. Similar to cysts, fibroblast-like cells were distributed throughout granuloma lesions, but no epithelial cell layer was evident.

ECM Genes Are Differentially Expressed in Granulomas and Cysts Compared to Healthy PDL

Out of 113 genes, 11 genes in periapical granulomas and 17 genes in cysts showed higher expression levels than in healthy PDL (Fig. 1). Of those overexpressed, ADAM metallopeptidase-1, integrin-β4, integrin-β7, laminin-α1, MMP-2, and tissue inhibitor of metalloproteinase-1 were similarly expressed in cysts and granulomas.

Extracellular matrix protein-1, integrin-α2B, MMP-10, MMP-7, integrin-αM, and laminin-β2 genes were more frequently expressed in cysts followed by a lower expression in granulomas, and the lowest expression was detected in healthy PDL. Osteonectin gene expression was similar in all tissues.

Integrin-α3, integrin-α5, and integrin-β1 in cysts and transforming growth factor β in granulomas were significantly elevated in lesions compared to healthy PDL. MMP-24 was detected in granulomas and cysts but not in healthy PDL, and ADAM metallopeptidase-13 and secreted phosphoprotein-1 were found exclusively in cysts but not in granulomas or healthy PDL. Catenin-α1, tissue inhibitor of metalloproteinase-3, and selectin-L were found exclusively in healthy PDL but not in periapical lesions; thus, these genes were suppressed in the lesions (Table 2). We selected for presentation only those genes that were up- or downregulated in cysts and granulomas. Relative expression for genes not modulated or genes not expressed were not reported.

Periapical Granulomas Exhibit High Gelatinolytic Activity Compared to Cysts

Given the high prevalence of MMPs and higher MMP-2 mRNA expression in both cysts and granulomas compared with healthy PDL, the presence of MMP activity in tissue sections was further investigated. *In situ* zymography revealed gelatinolytic activity in both cysts and granulomas. Gelatinolytic activity, detected in the

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