Modulation of Matrix Metalloproteinase 14, Tissue Inhibitor of Metalloproteinase 3, Tissue Inhibitor of Metalloproteinase 4, and Inducible Nitric Oxide Synthase in the Development of Periapical Lesions

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Abstract

Introduction: Periapical cysts and granulomas are chronic lesions caused by an inflammatory immune response against microbial challenge in the root canal. Different cell types, cytokines, and molecules have been associated with periapical lesion formation and expansion. Therefore, because of the chronic inflammatory state of these lesions, the aim of this study was to evaluate the *in situ* expression of matrix metalloproteinase (MMP)-14 and -19, tissue inhibitor of metalloproteinase (TIMP)-3 and -4, CD68, and inducible nitric oxide synthase (iNOS) in periapical cysts and granulomas. **Methods:** Sixteen cases of periapical cysts and 15 cases of periapical granulomas were analyzed. Ten normal dental pulps were used as the negative control. Immunohistochemistry was performed with anti–MMP-19, anti–MMP-14, anti–TIMP-3, anti–TIMP-4, anti-iNOS, and anti-CD68 antibodies. **Results:** The expression of TIMP-3, TIMP-4, iNOS, and CD68 was significantly higher in both the cyst and granuloma groups than in the control group. TIMP-4 was also significantly higher in cases of chronic apical abscess. There was also a significant difference in the expression of MMP-14 between the cyst and control groups. However, there were no differences in the expression of MMP-19 between the 3 groups. **Conclusions:** Our data suggest that the expression of MMP-14, TIMP-3, and TIMP-4 is associated with the development of periapical lesions. *(J Endod 2017;■:1–8)*

Key Words

Metalloproteinases, periapical lesions, tissue inhibitor of metalloproteinases

Significance

The expression of metalloproteinases seems to contribute to the degradation of the extracellular matrix in inflammatory processes and to bone destruction, formation and expansion of these lesions. Studies on metalloproteinases are important to understand the biology of periapical lesions.

Dental pulp diseases may lead to the development of lesions in the periradicular region, notably periapical granulomas and cysts (1). Both of them are chronic periapical lesions caused by a cell-mediated local immune response, inflammatory mediators, and cytokines against microbial change in order to inhibit the progression of root canal infection (2, 3). These lesions are characterized by the presence of mononuclear inflammatory infiltration consisting of lymphocytes, plasma cells, mast cells, and macrophages in the fibrous connective tissue (granulation tissue) (4).

Macrophages are the predominant cells in the inflammatory tissue of periapical lesions (5). They play a role in the protective response of these lesions as well as in the development and progression of the inflammatory response through the production of key cytokines (6). A specific marker of these cells is the $CD68^+$ molecule, which is present both in granulomas and cysts (7), and the prevalence of $CD68^+$ macrophages in these lesions has been evaluated in several studies (8–10).

A study showed that macrophages, lymphocytes, endothelial cells, and plasma cells can produce inducible nitric oxide synthase (iNOS) enzyme in periapical lesions (11), which, in turn, produce nitric oxide by arginine cleavage. Nitric oxide is a potent antimicrobial agent that can modulate the inflammatory process and that may even lead to tissue damage and expansion of periapical lesions (12).

Matrix metalloproteinases (MMPs) are zinc-dependent enzymes (endopeptidases) that degrade extracellular matrix components (13). They are produced by polymorphonuclear cells, monocytes, macrophages, fibroblasts, and mesenchymal cells, and they seem to play an important role in the development of cysts and periapical

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0099-2399/$ - see front matter

http://dx.doi.org/10.1016/j.joen.2017.02.020

granulomas (14) because they are associated with inflammation, bone remodeling, and the resorption process (15). Several MMPs have been associated with the development of periapical lesions, especially MMP-1, -2, -3 (16), and -13 (4, 15). However, the role of MMP-14 and MMP-19 has been poorly explored in this context. The former has been associated with collagenolytic activity (17) and is expressed during a pathological process related to bone resorption (18), whereas the latter degrades extracellular matrix components (19) and has been described in inflammatory diseases, such as rheumatoid arthritis and systemic lupus erythematosus (20).

MMPs are regulated by the tissue inhibitors of metalloproteinases (TIMPs) (21), which are proteins that regulate the activation ratios of MMPs as well as their ability to degrade certain substrates (22). The balance between the production of MMPs and TIMPs is necessary for the maintenance of homeostasis of the extracellular matrix with no pathological occurrence (23). The development and severity of periapical chronic diseases are mediated by an inflammatory immune response. Nonetheless, the growth mechanism and maintenance of the immune response in these diseases have not yet been fully explained in the literature. Therefore, the aim of this study was to evaluate the *in situ* expression of MMP-14 and -19, TIMP-3 and -4, CD68, and iNOS in periapical cysts and granulomas.

Materials and Methods

Sample

The sample consisted of 31 cases of periapical lesions (ie, 16 cases of periapical cysts and 15 of periapical granulomas) and 10 control cases. Patients were 10–64 years old and were diagnosed at the Pathology Department of the University of Uberaba School of Dentistry, Uberaba, Minas Gerais, Brazil, from January 2002 to January 2012.

The periapical lesions were diagnosed based on clinical and histopathological findings. A radicular cyst was defined as follows: a lesion in the periapical region of a nonvital tooth, a cavity containing fluid or semisolid material detected during surgery, and histologic evidence of a stratified nonkeratinized squamous epithelium completely or partially lining a cystic cavity or tissue fragments. For dental granulomas, in addition to the previously mentioned clinical features, a chronic inflammatory reaction in the biopsy specimen detected during histopathological analysis was also observed (24). Samples were collected during surgery at the Dental Clinic of University of Uberaba, Uberaba, Minas Gerais, Brazil.

Ten dental pulps were selected from healthy impacted third molars with surgical indication and were used as healthy controls. All fragments were fixed in 10% formalin and embedded in paraffin. Paraffin blocks were cut in 5-μm serial sections for subsequent histopathological and immunohistochemical analysis.

The study was approved by the Research Ethics Committee of the University of Uberaba under the Certificate of Presentation for Ethical Consideration (no. 0003.0.227.000-6).

Immunohistochemistry

For immunohistochemistry, the samples were deparaffinized in xylene and alcohol baths and then immersed in phosphate-buffered saline. For antigen retrieval and endogenous peroxidase inhibition, the sections were treated with 3% hydrogen peroxide in methanol for 10 minutes and then incubated in citrate buffer (0.01 mol/L, pH = 6.0) for 30 minutes at 90°C. Then, they were incubated in 2% bovine serum albumin for 30 minutes at room temperature to reduce nonspecific binding. After that, the samples were individually incubated with the following human-specific primary antibodies: anti–MMP-19 (1:20) (AF6790; R&D Systems, Minneapolis, MN, USA), anti–MMP-14 (1:20) (MAB918, R&D Systems), anti–TIMP-3 (1:20) (MAB973, R&D), anti–TIMP-4 (1:20) (MAB974, R&D), anti-iNOS (1:300) (ab3523; Abcam, Cambridge, MA), and anti-CD68 (1:50) (sc-52998; Santa Cruz Biotech, Dallas, TX) and diluted before use in 2% bovine serum albumin for 2 hours at 37°C. Then, the samples were incubated with secondary antibody (LSAB + System-HRP Kit, RB/Mo/Goat, K0690-1; Dako, Carpinteria, CA) and conjugated to biotin (Biotinylated Link Universal and Streptavidin-HRP, Dako) for 30 minutes at 37°C. The samples were then washed and incubated with streptavidin-peroxidase conjugate for 30 minutes at room temperature. The reaction was developed by incubation with diaminobenzidine (Sigma-Aldrich, St Louis, MO). Finally, the sections were counterstained with hematoxylin. A negative control reaction was performed by omitting the primary antibody.

Histologic analysis of the slides was performed using an ordinary light microscope, and morphologic analysis of the positive cells of each tested cytokine was performed in a quantitative manner.

Morphometric Analysis

Morphometric analysis was performed through quantification of the number of immunopositive cells. Analysis of MMPs and TIMPs was performed from the histologic images captured with a digital system and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD). For this purpose, each field to be quantified was captured with a camera coupled to the microscope and a computer for image scanning. For analysis of immunopositivity for iNOS and CD68, the cells were counted in the microscope.

The number of cells in each field was determined as well as the number of fields and the area of each field. The density of positive cells was expressed by the number of cells per mm^2. The density of positive cells was expressed by the number of positive cells divided by the area of each field multiplied by the number of fields.

Statistical Analysis

Data were analyzed using GraphPad Prism 5.0 program (GraphPad Software, La Jolla, CA). After normality testing using the Shapiro-Wilk test and analysis of variance (ANOVA), the Student *t* test, ANOVA with the Tukey post hoc test, or the Kruskal-Wallis test with the Dunn post hoc test were used. Correlations between 2 continuous variables with normal distribution were assessed by Pearson correlation analysis (r).

The continuous variables with normal distribution were expressed as the mean and standard deviation, whereas those with non-normal distribution were expressed as the median with minimum and maximum values and percentiles. The results were considered statistically significant when $P < .05$.

Results

We evaluated 41 cases (24 women and 17 men with an average age of 35.07 years), 31 cases with periapical lesions and 10 control subjects. Sixteen of these 31 individuals were diagnosed with periapical cysts, and 15 were diagnosed with granulomas. Moreover, pathological examination showed that these periapical lesion cases were exacerbated in 13 subjects and that polymorphonuclear cells prevailed. In the control group, 10 samples were obtained from the pulp tissue of healthy third molars. For these subjects, morphologic analysis showed that the pulps had no any signs of inflammation (Fig. 1*A*). Representative images of the inflammatory infiltrate in the cyst group and granuloma group are shown in Figure 1*B* and *C*, respectively.

As for the analysis of positive cells for MMPs, the expression of MMP-14 was significantly higher in cyst cases than in the control group (Kruskal-Wallis, $P < .05$) (Fig. 1*D–G*). The expression of MMP-19 was