Immunoistochemical expression of TGF-β1 and MMP-9 in periapical lesions

Abstract: The objective of this study was to evaluate the expression of matrix metalloproteinase 9 (MMP-9) and transforming growth factor beta (TGF-β1) in periapical lesion samples correlated with the intensity of the inflammatory infiltrate and thickness of the epithelial lining. Forty-five cases of periapical lesions (23 periapical granulomas and 22 radicular cysts) were subjected to morphological and immunohistochemical analyses using anti-MMP-9 and anti-TGF-β1 antibodies. The data were analyzed using the following tests: non-parametric Mann-Whitney, chi-square, Fisher’s exact test and Spearman’s correlation test (P<0.05). Analysis of inflammatory infiltrate revealed that 78% of periapical granulomas presented infiltrate grade III, in contrast with 32% of radicular cysts (P<0.001). Morphological evaluation of the epithelial thickness in radicular cysts revealed the presence of atrophic epithelium in 86% of the cysts. The immunostaining of MMP-9 was score 2 in 67% of the granulomas and 77% of the cysts. Both lesions were predominantly score 1 for TGF-β1. Significant differences were confirmed between the expression scores of TGF-β1 and MMP-9 in periapical granulomas and radicular cysts (p = 0.004) and in radicular cysts (p < 0.001). Expression of TGF-β1 was different for periapical granulomas and radicular cysts. This immunoregulatory cytokine seems more representative in asymptomatic lesions. The extracellular matrix remodeling process dependent on MMP-9 seems to be similar for both periapical granulomas and radicular cysts. TGF-β1 and MMP-9 may play an important role in the maintenance of periapical lesions.

Keywords: Inflammation; Immunohistochemistry; Periapical Granuloma; Radicular Cyst; Biopsy.

Introduction

Periapical granulomas and radicular cysts are osteolytic inflammatory lesions affecting the jaws, and representing up to two-thirds of all radiolucent periapical lesions. They develop in periapical tissues as a direct immunological response mainly to the progression and maintenance of endodontic bacterial infection. In addition, mechanical and/or thermic injuries that may result in pulp necrosis, as well as infection by human cytomegalovirus and Epstein-Barr virus, may also be implicated as etiological factors in a small number of cases.

When an immune/inflammatory response is maintained in periapical tissues, it can lead to the accumulation of lymphocytes, plasma cells,
macrophages —mediating the scarce neutrophils — and eosinophils, resulting in a well-circumscribed lesion called periapical granuloma.4,5 On the other hand, continuous and chronic inflammation, resulting from the host immune system acting to fully eradicate infection, may reactivate the epithelial cell rests of Malassez and other epithelial sources that proliferate to form radicular cysts.6,7

Several immune/inflammatory mediators and proteases may be involved in the formation of periapical granulomas and radicular cysts, such as transforming growth factor-β1 (TGF-β1) and matrix metalloproteinase-9 (MMP-9).8,9 TGF-β1 is a polypeptide member of the transforming growth factor-β superfamily of cytokines, which is secreted by platelets and inflammatory cells. It performs many cellular functions, including the control of cell growth, cell proliferation, cell differentiation, apoptosis and inflammation, as well as tissue repair.10 Additionally, TGF-β1 immune/inflammatory functions include (a) chemotaxis for monocytes, neutrophils and fibroblasts; (b) initiation, growth and differentiation of inflammatory cells; and (c) formation of bone structures.6

Several cells, such as neutrophils, macrophages, T cells, mast cells and odontoblasts, may secrete MMP-9, a metalloenzyme belonging the gelatinase group of matrix metalloproteinases (MMPs) that degrade a vast number of extracellular matrix (ECM) components, including denatured collagen, basement membrane and bone matrix. These MMPs are essential for the initiation of bone resorption, and also participate as modulators of acute and chronic inflammatory responses, since they can activate both proinflammatory and immunoregulatory (such as TGF-β1) mediators.11,13,16,17,18

Despite controversial evidence implicating proinflammatory modulators in the pathogenesis of periapical granulomas and radicular cysts, little is known about their immunoregulatory and modulatory mechanisms. This study descriptively analyzed and verified possible correlations between the immunohistochemical expression of TGF-β1 and MMP-9 in periapical lesions and clinical/morphological variables in an endeavor to gain a better understanding of the pathogenesis of periapical lesions, and confirm protein expression related to progression.

Methodology

Study design and tissue sample
The study included a descriptive analysis of the immunohistochemical expression pattern of TGF-β1 and MMP-9 in forty-five randomly selected cases of periapical lesions, including 23 periapical granulomas and 22 radicular cysts. Data related to the patients (age and gender) and their clinical signs (symptomatology, radiographic findings and anatomical site) were collected through biopsy records, and information on the immunoexpression of TGF-β1 and MMP-9 was obtained after performing the experimental study. The selection and retrieval of all cases, their biopsy records and laboratorial experiments were performed at the Surgical Pathology Laboratory of the University of Pernambuco, Brazil. The study was approved by the Research Ethics Committee of the University of Pernambuco (0082.0.097.000-10).

Morphological analysis
Five-micrometer-thick tissue sections obtained from paraffin-embedded blocks of periapical granulomas and radicular cysts were placed on a glass slide, stained with hematoxylin-eosin and visualized under light microscopy. The intensity of the inflammatory infiltrate was evaluated according to the method adopted.19,20 Each specimen was graded according to its inflammatory condition, in three consecutive microscopic fields, starting with the inner portion of the specimen and proceeding deeper into the connective tissue. In brief, each specimen was graded at 400X magnification as follows:

1. Grade I, inflammatory cells restricted to the first microscopic field;
2. Grade II, inflammatory cells extending to the second microscopic field;
3. Grade III, inflammatory cells in all three microscopic fields.

The thickness of the epithelial lining was defined as atrophic (2-10 cell layers and flat epithelial/capsule boundary) or hyperplastic (> 10 cell layers and undulating epithelial/capsule boundary, often
arranged into proliferating arcades), based on the predominant pattern found in each case.\textsuperscript{20}

**Immunohistochemical method**

The immunohistochemical study was performed by obtaining 3-mm-thick sections from paraffin-embedded tissue blocks and placing them on a silanized glass slide. The sections were deparaffinized, then washed in phosphate-buffered saline (PBS) and submitted to antigen retrieval (Table 1). Afterwards, the samples were immersed in 3% hydrogen peroxide to block endogenous peroxidase activity. After treatment with normal serum, the sections were incubated with the anti-TGF-β1 and anti-MMP-9 primary antibodies (Table 1) in a moist chamber at room temperature, according to the streptavidin-biotin peroxidase method optimized by LSAB amplification system (Streptavidin biotin-labeled primary mouse antibodies, DAKO, Carpinteria, CA, USA). Peroxidase activity was visualized by immersing the tissue sections in dianinobenzidine (Liquid DAB Substrate, DAKO), which resulted in a brown reaction product. Finally, the sections were counterstained with Harris hematoxylin and coverslipped. Sections of human breast carcinoma were used as positive controls for the anti-TGF-β1 antibody, and sections of spleen tissue were used for the anti-MMP-9 antibody. As negative controls, the samples were treated as described previously, except that the primary antibody was omitted and replaced by non-immune murine IgG1 (X-0931, DAKO) or 1% BSA-PBS for both antibodies studied.

**Immunostaining assessment and statistical analysis**

Immunohistochemical analysis was performed by two oral pathologists with a Nikon E200 light microscope (Nikon, Tokyo, Japan). Tissue sections were examined under light microscopy (100X magnification) to identify areas containing the largest number of immunoreactive cells, and the selected microscopic fields were then analyzed at 400X magnification. The following aspects were considered in evaluating the TGF-β1 and MMP-9 immunostaining: presence (+) or absence (-) of immunostaining, type of immunopositive cells and their tissue distribution (focal or diffuse). Immunostaining scores were attributed semi-quantitatively to the percentage of positive cells in each case: score 0 - no positive cells; score 1 - 1-50% positive cells; and score 2 - > 50% positive cells.

A descriptive statistical analysis was conducted using Windows Office Excel 2015\textregistered. WinPepi software for Windows, version 11.32, 2013, was used to test the possible differences and/or inferential statistical associations between the immunostaining scores in periapical granulomas and radicular cysts, using the non-parametric Mann-Whitney, Pearson’s chi-square, Fisher’s exact tests and Spearman’s correlation test. The confidence interval was defined as 95% and a value of $p < 0.05$ was considered statistically significant.

**Results**

**Analysis of clinical and radiographic aspects**

An analysis of the 23 cases of periapical granuloma showed a higher incidence found in female patients ($n = 17; 72\%$), aged between 30 and 40 years ($n = 14; 60\%$), asymptomatic ($n = 15; 64\%$), showing radiolucencies ($n = 22; 96\%$), and anatomically sited mostly in the anterior maxilla ($n = 16; 68\%$). The 22 radicular cysts were more prevalent in male patients ($n = 12; 56\%$), aged between 20 and 30 years ($n = 12; 53\%$), with no symptoms ($n = 18; 82\%$). As for the radiographic appearance and anatomical location, a radiolucent image was observed in 21 cases ($96\%$), mostly in the anterior region of the maxilla ($n = 13; 60\%$). In relation to the clinical characteristics and radiographic findings of periapical granulomas and radicular cysts, the only significant differences were found in relation to gender ($p < 0.001, 95\% CI.: 1.81 to 5.92$; Pearson’s

**Table 1.** Manufacturer, clone, antigen retrieval, dilution, and incubation period of the monoclonal primary antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Clone</th>
<th>Antigen retrieval</th>
<th>Dilution</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>Abcam</td>
<td>Ab79781</td>
<td>Pepsin 1%, pH 1.8, Pascal, 37°C, 3 min</td>
<td>01:50</td>
<td>60 min</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Santa Cruz Biotechnology</td>
<td>Sc-146</td>
<td>Citrate, pH 6.0, Pascal, 37°C, 3 min</td>
<td>0.128472222</td>
<td>30 min</td>
</tr>
</tbody>
</table>
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chi-square) and symptoms (p = 0.004, 95%CI: 0.20 to 0.75; Pearson’s chi-square).

Morphological analysis

Analysis of inflammatory infiltrate in periapical granuloma revealed 18 cases (78%) with inflammatory infiltrate grade III, 3 cases (13%) with grade II and 2 cases (9%) with grade I. In radicular cysts, 5 cases (23%) had inflammatory infiltrate grade III, 7 cases (32%) had grade II, and 10 cases (45%) had grade I. The non-parametric Mann-Whitney test revealed a significant difference between the degree of inflammatory infiltrate in periapical granulomas versus radicular cysts (p < 0.001). Morphological evaluation of the epithelial thickness in radicular cysts revealed the presence of an atrophic epithelium in 19 cases (86%) and a hyperplastic epithelium in 3 cases (14%). The degree of inflammatory infiltrate and epithelial thickness was not significantly correlated to any clinical or radiographic finding for the periapical lesions examined.

Immunohistochemical assessment

The immunohistochemical expressions of TGF-β1 and MMP-9 were distributed diffusely in all the cases of both periapical granuloma and radicular cysts (Figure 1) (Table 2). In the periapical granulomas, the inflammatory cells and the stromal connective tissue exhibited positivity to both proteins studied, whereas the epithelial cystic lining (atrophic or hyperplastic), as well as the inflammatory cells and connective tissue of the cystic capsule in radicular cysts, showed cellular positivity. Nevertheless, there were no significant associations and/or differences in the immunoexpression of TGF-β1 and MMP-9, in relation to clinical signs, radiographic findings,

Figure 1. Representative photomicrographs of immunohistochemical staining of the studied markers in periapical lesions. (a) MMP-9 expression in radicular cysts (IHC, 200X), (b) MMP-9 expression in periapical granuloma (IHC, 100X), (c) TGF-β1 expression in radicular cysts (IHC, 100X), (d) TGF-β1 expression in periapical granuloma (IHC, 100X).
and level of inflammatory infiltrate visualized in the periradicular lesions evaluated.

The majority of periapical granulomas had score 1 for TGF-β1 immunoexpression (n = 12; 54%), and score 2 for MMP-9 (n = 16; 67%). Similarly, in radicular cysts, score 1 was more prevalent for TGF-β1 (n = 15; 68%), whereas score 2 was higher for MMP-9 (n = 17; 77%) (Table 2). Significant differences could be seen between the expression scores of TGF-β1 and MMP-9 in periapical granulomas (p = 0.004; 95%CI: 1.34–4.24) and radicular cysts (p < 0.001; 95%CI: 0.31–0.58). Notwithstanding, when the immunohistochemical expression of each protein was evaluated in the periapical lesions, only TGF-β1 revealed relevant differential expression between these lesions (Pearson’s chi-square: p = 0.042; CI: 0.31–0.99) (Table 2).

In periapical cysts, no significant correlation (Spearman’s correlation test) was observed between TGF-β1 and MMP-9 (r = 0.370; p = 0.09). As for periapical granulomas, a significant moderate positive correlation (Spearman’s correlation test) was verified between these proteins (r = 0.633; p = 0.001).

### Discussion

The development of periapical lesions is a complex and dynamic process in which multiple types of inflammatory cells and their derivatives are involved, but the details are not well understood. Substantial evidence suggests that these lesions are the result of a long-term endodontic infection or pulp necrosis leading to a progressive immune/inflammatory response, whose persistence in periapical tissues can result in the resorption of the surrounding bone and reactivation of epithelial cell rests of Malassez10,21,22; which are probably the main source of the epithelial lining of radicular cysts.9 In fact, it seems that this persistent, chronic inflammation and destruction of bone depends on the inability of a host defense to eradicate the infection.10

The participants in this study were predominantly females, aged 20-40 years, and exhibited a well-demarcated radiolucency image, and asymptomatic lesions affecting the anterior maxilla. This patient profile was similar to that of several other recent epidemiological reports.23,24 However, only gender and symptoms revealed differences between periapical granulomas and radicular cysts; however, these differences could not be correlated with other clinical variables or with the TGF-β1 and MMP-9 immunohistochemical assessments. The results suggest that there is no specific relationship between the clinical, demographic and immunohistochemical features of the periapical lesions examined.
The morphological analysis showed that periapical granulomas exhibited higher grades of inflammatory infiltrate than the cysts, but this significant difference was not associated with the scores attributed to the immunohistochemical expression of TGF-β1 and MMP-9. In contrast, Gazivoda et al.\textsuperscript{10} used a fluorescent bead immunoassay and/or ELISA to prove that immunoregulatory cytokines such as TGF-β are more important for suppression of inflammation in asymptomatic lesions. Based on this finding, the effect of TGF-β was stronger than that of IL-10. In addition, no differences were established in the present study between the epithelial thickness of radicular cyst lining and the immunoexpression of TGF-β1 and MMP-9, as corroborated by other authors.\textsuperscript{25,26}

In this research, the TGF-β1 expression revealed differences between periapical granulomas and radicular cysts, thus corroborating the observation by Marçal et al.\textsuperscript{27} and Teixeira-Salum et al.\textsuperscript{28}. Although the data could not confirm any significant association between symptoms and TGF-β1 expression, their descriptive analysis showed that the majority of granulomas were asymptomatic and presented a greater number of cases with higher TGF-β1 scores (score 2). Divergently, there were more cases of cysts with symptoms, but fewer cases with higher scores for this protein (Table 2). Thus, these results corroborate those of other authors, demonstrating that asymptomatic periapical lesions exhibited higher levels of TGF-β1 and other immunoregulatory cytokines.\textsuperscript{10,21}

Furthermore, substantial evidence suggests that asymptomatic periapical lesions expressing high levels of immunoregulatory cytokines are less immunologically active.\textsuperscript{3,10}

The MMP-9 immunohistochemical expression was not different among the periapical lesions evaluated in this study. Notwithstanding, an immunohistochemistry assay by Carneiro et al.\textsuperscript{29} found that the non-epithelialized periapical lesions exhibited higher MMP-9-positive cells, but no significant differences were observed among these lesions. Andrade et al.\textsuperscript{26} also evidenced a higher expression of MMP-9 in granulomas than in radicular and residual radicular cysts. These data suggest that MMP-9 presents a similar remodeling activity of extracellular matrix in periapical lesions. Additionally, the expression of MMP-9 was not relevant concerning the symptoms and grade of inflammatory infiltrate of the lesions. These results are corroborated by Faustino et al.\textsuperscript{30} and Andrade et al.\textsuperscript{26}, who showed that the MMP-9 immunoexpression was no different between symptomatology and grade of inflammation among periapical granulomas, radicular cysts and residual radicular cysts.

Nevertheless, MMP-9 showed significantly higher expression scores than TGF-β1, both in periapical granulomas and radicular cysts. MMP-9 is a gelatinase that plays an important role in bone resorption, because of its ability to degrade the denatured collagen of the extracellular matrix, and also activates uncleaved TGF-β in tissues.\textsuperscript{16,17,29,30,31}

A long-term inflammation maintained in periapical tissues could increase the levels of MMP-9, inducing the remodeling of bone and activation of TGF-β, which is important to avert osteoclast formation and stimulate tissue repair mechanisms.\textsuperscript{2,3,31,33} Particularly noteworthy is that expressions of TGF-β1 and MMP-9 may depend on the secretion of other proteins, such as cytokines, growth factors, transcriptional factors, other matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases (TIMPs).\textsuperscript{2,3,4,5,6,31,32,33,34,35,36} Experiments using MMP-9 knockout mice\textsuperscript{14} evidenced that these animals exhibited larger periapical lesions with greater inflammation, indicating a central participation of MMP-9 in the development of periapical lesions. The presence of TGF-β1 and MMP-9 in periapical lesions denotes their important role in the maintenance, development and exacerbation of chronic processes,\textsuperscript{5,14,34,35} although further studies are needed to evaluate the possible molecular mechanisms involved in the progression of these lesions.

**Conclusions**

TGF-β1 expression varied between periapical granulomas and radicular cysts. This immunoregulatory cytokine seems to be more representative in asymptomatic lesions. The extracellular matrix remodeling process dependent on MMP-9 seems to be similar in both periapical granulomas and radicular cysts, although it
may be influenced by TGF-β1. There was no difference and/or association between TGF-β1 and MMP-9 immunoexpression in respect to clinical or radiographic results, grade of inflammatory infiltrate and type of cystic epithelial lining. TGF-β1 and MMP-9 may be factors involved in the maintenance of periapical lesions.

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References