Immunohistochemical analysis of FoxP3+ cells in periapical granulomas and radicular cysts

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ABSTRACT
Objectives: To compare the number of FoxP3+ cells between periapical granulomas (PGs) and radicular cysts (RCs), and to correlate this number with the intensity of the inflammatory infiltrate in these lesions and with epithelial thickness of RCs.

Study design: Thirty PGs and 30 RCs were submitted to immunohistochemical analysis using an anti-FoxP3 polyclonal antibody. FoxP3+ cells were counted under a light microscope (×400 magnification) in five fields and the mean value was calculated for each specimen. Statistical tests were used to evaluate differences in the number of FoxP3+ cells according to type of lesion (PG vs. RC), intensity of the inflammatory infiltrate (grade I/II vs. grade III), and epithelial thickness of RCs (atrophic vs. hyperplastic).

Results: FoxP3+ cells were detected in most PGs (93.3%) and RCs (93.3%). The median number of FoxP3+ cells was 2.40 in PGs and 1.00 in RCs, with this difference being statistically significant (P = 0.005). No significant differences in the number of FoxP3+ cells were observed in terms of the intensity of the inflammatory infiltrate (P = 0.465) or epithelial thickness of RCs (P = 0.737).

Conclusions: The present results suggest a greater participation of regulatory T cells in the modulation of the inflammatory response in PGs. In addition, the presence of a less effective regulatory environment in RCs, together with the high levels of inflammatory mediators as reported in the literature, may contribute to the greater growth potential of these lesions.

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1. Introduction

Periapical lesions, which include periapical granulomas (PGs) and radicular cysts (RCs), can arise after pulp necrosis as a consequence of the immune response to continuous antigen stimulation by microorganisms present in the root canals.1-3 Persistent inflammation at the periapical tissues is associated with resorption of adjacent bone, which is replaced with fibrovascular connective tissue, forming a PG. The latter is a secondary and defensive reaction of the host in an attempt to control progression of the infectious process.4 As a consequence of these immunopathological responses, the epithelial rests of Malassez are stimulated to proliferate, an event that can result in the development of a RC.5,6

Factors such as the amount and type of cytokines, antigen nature and the adequate expression of receptors and costimulatory molecules, as well as the type of antigen-presenting cells and genetic constitution of the host, modulate the progression and severity of inflammatory processes and

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influence the type of host response (T-helper 1 [Th1] or T-helper 2 [Th2]). Although Th1/Th2 responses are induced by cytokines, the two types of effector responses are regulated by a heterogeneous family of cells, known as regulatory T (Treg) cells. These cells play a major role in the modulation of immune responses, induction and maintenance of immune tolerance, and also in the prevention of autoimmune diseases. The mechanism of action of Treg cells involves direct suppression of the activation of T and B lymphocytes and natural killer (NK) cells through cellular mechanisms mediated by surface molecules such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), or by the synthesis of immunosuppressive cytokines such as interleukin 10 (IL-10) and transforming growth factor beta (TGF-β). The basic function of this protein is to regulate the activity of these cells.

Studies investigating the presence and function of Treg cells in periapical lesions are scarce in the literature. Therefore, the objective of the present study was to compare the number of FoxP3+ cells between PGs and RCs, and to correlate this number with the intensity of the inflammatory infiltrate in these lesions and with epithelial thickness of RCs. These data should contribute to a better understanding of the immunopathogenesis and the consequent progression and severity of periapical lesions.

2. Materials and methods

Sixty specimens, including 30 PGs and 30 RCs, obtained from the Oral Pathology Department of the Federal University of Rio Grande do Norte (UFRN) were randomly selected for this study. All PGs and RCs were obtained from human teeth without endodontic treatment. Pathological diagnoses were confirmed on the basis of clinical, radiographic and histological criteria. Only PGs without odontogenic epithelium were selected. Moreover, all RCs presented unequivocal cystic cavity lined by odontogenic epithelium. The cases were not matched for age, gender, or anatomic location. The study was approved by the Research Ethics Committee of UFRN, Natal, Brazil (protocol number: 056/10).

2.1. Morphological analysis

For the morphological analysis, 5-μm-thick tissue sections were stained with haematoxylin and eosin. The intensity of the inflammatory infiltrate was evaluated according to an adaptation of the method proposed by Tsi et al. Grading of each specimen was recorded on the inflammatory condition in 1 microscopic field, starting from the inner portion of the specimen and proceeding deeper into connective tissue. Briefly, each specimen was graded at ×200 magnification as: grade I, inflammatory cells less than one-third; grade II, inflammatory cells between one-third and two-thirds; and grade III, inflammatory cells more than two-thirds. Analysis of the thickness of the epithelial lining in RCs was performed considering all the cyst lining. 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 in each case.

2.2. Immunohistochemical methods

For the immunohistochemical study, 3-μm-thick sections were obtained from paraffin-embedded tissue blocks. The tissue sections were deparaffinized and immersed in 3% hydrogen peroxide to block endogenous peroxidase activity. Antigen retrieval was performed in a Pascal pressure cooker with citrate buffer, pH 6.0. After treatment with normal serum, the sections were incubated with the primary anti-FoxP3 polyclonal antibody diluted 1:150 (clone H190, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 18 h. The tissue sections were then washed twice in PBS and treated with immunoperoxidase-based kit (EnVisionTM + Dual Link System-HRP; Dako, Carpinteria, CA, USA) at room temperature to bind the primary antibody. Peroxidase activity was visualized by immersing tissue sections in diamobenzidine (Liquid DAB + Substrate; Dako, Carpinteria, CA, USA), resulting in a brown reaction product. Finally, the tissue sections were counterstained with Mayer haematoxylin and coverslipped. Sections of normal human tonsil were used as positive control for FoxP3. As negative control, samples were treated as earlier, except that the primary antibody was replaced by a solution of bovine serum albumin in PBS.

2.3. Immunostaining assessment and statistical analysis

After the immunohistochemical treatment, the tissue sections were examined by two observers under a light microscope (Olympus CX31, Olympus Japan Co., Tokyo, Japan). Using ×100 magnification, five fields with the largest number of immunostained cells were identified. In these fields, FoxP3+ cells were counted at ×400 magnification and the total number of positive cells per case was calculated, permitting the calculation of the mean number of FoxP3+ cells per specimen.

The results obtained were submitted to statistical analysis. Computations were made using the Statistical Package for the Social Sciences (version 17.0; SPSS Inc., Chicago, IL, USA). For the intensity of the inflammatory infiltrate, lesions showing inflammatory infiltrate grades I and II were combined into one group and compared with those presenting inflammatory infiltrate grade III. The Kolmogorov–Smirnov test revealed absence of a normal distribution for the number of FoxP3+ cells. Therefore, to compare the number of FoxP3+ cells according to type of lesion (PGs vs. RCs), thickness of the epithelial lining (atrophic vs. hyperplastic), and intensity of inflammatory infiltrate (grades I/II vs. grade III), the Mann–Whitney non-parametric test was performed. Spearman’s correlation test was performed to verify possible correlations between the number of FoxP3+ cells and the intensity of the inflammatory infiltrate. For all tests, significance level was set at 5% (P < 0.05).
3. Results

3.1. Morphological analysis

Analysis of the inflammatory infiltrate in PGs revealed all cases (n = 30; 100%) with inflammatory infiltrate grade III. In RCs, 16 cases (53.3%) showed inflammatory infiltrate grade III, 13 cases (43.3%) inflammatory infiltrate grade II, and only 1 case (3.3%) inflammatory infiltrate grade I. Morphological analysis of the epithelial thickness in RCs revealed the presence of an atrophic epithelium in 25 cases (83.3%) and a hyperplastic epithelium in only 5 cases (16.7%).

3.2. Immunohistochemical analysis

Analysis of the immunohistochemical expression of anti-FoxP3 antibody showed the presence of FoxP3+ cells in 28 PGs (93.3%) and in 28 RCs (93.3%). The median number of FoxP3+ cells was 2.40 (0.00–9.60) in PGs (Fig. 1A) and 1.00 (0.00–5.00) in RCs (Fig. 1B). The non-parametric Mann–Whitney test disclosed a statistically significant higher median number of FoxP3+ cells in PGs when compared to RCs (P = 0.005) (Table 1).

Since no case of PG with inflammatory infiltrate grade I or II was detected in this study, analysis of the number of FoxP3+ cells according to intensity of the inflammatory infiltrate was performed only in RCs. The median number of FoxP3+ cells was 1.20 (0.00–5.00) in lesions with inflammatory infiltrate grade III (Fig. 2A) and 1.00 (0.20–4.40) in lesions with inflammatory infiltrate grade I/II (Fig. 2B). Comparison of the median number of FoxP3+ cells in relation to the intensity of the inflammatory infiltrate revealed no statistically significant difference between groups (P = 0.465) (Table 1). In addition, Spearman’s correlation test disclosed no significant correlation between the number of FoxP3+ cells and the intensity of the inflammatory infiltrate (r = –0.090; P = 0.635).

With respect to the thickness of the epithelial lining, the median number of FoxP3+ cells was 1.40 (0.20–2.80) in lesions with hyperplastic epithelium and 1.00 (0.00–5.00) in lesions with atrophic epithelium. The non-parametric Mann–Whitney test revealed no statistically significant differences in the median number of FoxP3+ cells between RCs with atrophic
epithelium and RCs with hyperplastic epithelium (P = 0.737) (Table 1).

4. Discussion

Periapical lesions develop in response to chronic antigenic stimulation of periapical tissue caused by bacterial infection in root canal.13 As a consequence, innate and acquired defence mechanisms are activated, which comprise vascular and cellular events that culminate in alveolar bone resorption in the apex of the tooth.23 Different cells are involved in this process, including neutrophils, macrophages, NK cells, Langerhans cells, and T and B lymphocytes, as well as Treg cells which regulate the immune response.5,24 However, the role of Treg cells in the modulation of the immune response in PGs and RCs has not been fully established.9,19

According to Salomon et al.25 a combination of strong antigen signals and maximum costimulation is necessary for the development of Treg cells. This condition is easily found in periapical lesions due to the strong antigen stimulation arising from the root canals,1,5,9,13,19 a fact explaining the presence of FoxP3+ cells in most PG (93.3%) and RC (93.3%) specimens studied here.

The present immunohistochemical study evaluated the number of FoxP3+ cells in PGs and RCs. The results showed a significantly larger number of FoxP3+ cells in PGs compared to RCs (P = 0.005). Similarly, Fukuda et al.10 investigating the mRNA expression of FoxP3 and of cytokines involved in Th1 (T-bet, interferon-γ [IFN-γ]) and Th2 (GATA-3, IL-4, IL-10) responses by real time-PCR in RCs and PGs, observed higher expression of FoxP3 and IL-10 in the latter. According to these authors, Treg cells play an important role in the modulation of the inflammatory microenvironment of periapical lesions. The authors suggested a possible suppression of the Th1 response in PGs. On the other hand, Marçal et al.3 evaluating the number of FoxP3+ cells in RCs and PGs, found no significant differences between these lesions.

Proinflammatory cytokines such as IFN-γ and tumour necrosis factor alpha (TNF-α) regulate Th1 responses, whereas IL-4 is the key cytokine involved in Th2 responses. These mediators trigger inflammatory responses that result in the progression of periapical lesions.2,3,26 These immune responses are modulated by FoxP3+ cells through direct cellular mechanisms, mediated by CTLa-4,7,11,16,27 or through indirect mechanisms, mediated by the synthesis of TGF-β and IL-10.4,7,9,13,27,28 In a comparative study of PGs and RCs, Teixeira-Salum et al.8 detected higher expression of TGF-β in PGs, and of IL-4, IFN-γ and TNF-α in RCs. Taken together, the results reported by these authors and the present findings suggest a more effective participation of FoxP3+ cells in the modulation of inflammatory responses in PGs, which can be explained by the high expression of TGF-β, a molecule suppressing Th1 and Th2 responses, and by the low levels of proinflammatory mediators regulating Th1 (IFN-γ and TNF-α) and Th2 (IL-4) responses in these lesions. Also within this context, the smaller number of FoxP3+ cells in RCs in comparison with PGs, observed in the present study, together with the high levels of inflammatory mediators and low levels of TGF-β in these lesions reported in other studies,8,10 may be related to the existence of a minimal regulatory environment in RCs that may contribute to the greater growth potential of these lesions.2,9,29–31

FoxP3+ cells have been studied in diseases of different aetiology, such as rheumatoid arthritis,22 atopic dermatitis,23 lichen planus,24 and intestinal inflammatory disease.35 In an animal model of experimental periodontitis, Garlet et al.37 observed increased levels of Treg cells and high expression of immunosuppressive cytokines (CTLa-4, TGF-β, and IL-10). The authors also found that animals treated with anti-glucocorticoid-inducible TNF receptor (GITR), an inhibitor of Treg cell function, exhibited a marked increase in bone loss and inflammatory cell migration, as well as a decrease in the expression of immunosuppressive cytokines. In agreement with these findings, Nakajima et al.9 suggested that in gingivitis Treg cells control the immunopathological process by preventing the destruction of periodontal bone. On the other hand, in established periodontitis Treg cells are recruited to suppress bone destruction through putative autoimmune mechanisms via a negative feedback system.8

It has been suggested that the higher the intensity of the inflammatory infiltrate, the higher the antigen stimulation and consequent tissue damage.30 On the other hand, immune surveillance by Treg cells would limit the tissue damage provoked by the host immune response and by the direct action of the microorganisms.27,37 Despite those reports, in the present study, no significant difference in the number of FoxP3 cells was observed in terms of the intensity of the inflammatory infiltrate of RCs (P = 0.465). In addition, no significant correlation was found between the number of FoxP3 cells and the intensity of the inflammatory infiltrate (P = 0.635). Thus, immune surveillance by Treg cells does not seem to be more effective in RCs with intense inflammatory infiltrates.
The status of the lining epithelium (atrophic or hyperplastic) has been described as a histological parameter indicating growth activity/inactivity in RCs. In quiescent lesions (atrophic epithelium), no cystic growth is observed despite the presence of antigens and enzymes able to induce immune responses, epithelial proliferation, and bone resorption. During this phase, the growth of RCs is probably limited by immunosuppressive responses. Nevertheless, no significant difference in the number of FoxP3+ cells was observed between RCs with hyperplastic and atrophic epithelium (P = 0.735). In view of the small number of RCs with hyperplastic epithelium (n = 5) in the present study, further investigations are needed to identify possible differences in the immune response between active (hyperplastic epithelium) and quiescent (atrophic epithelium) RCs.

In conclusion, the present results demonstrated the presence of FoxP3+ cells in inflammatory periapical lesions and a larger number of these cells in FGs compared to RCs, suggesting a greater participation of Treg cells in the modulation of the inflammatory response in the former. In addition, the number of FoxP3+ cells was not intimately correlated with the intensity of the inflammatory infiltrate or with the thickness of the lining epithelium in RCs. It is suggested that the presence of a less effective regulatory environment in RCs, associated with high levels of inflammatory mediators as reported in the literature, contribute to the greater growth potential of these lesions.

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Competing interests

None declared.

Ethical approval

The ethical approval for our research was given by the Ethics Committee of the Federal University of Rio Grande do Norte, Brazil (protocol number: 056/10).

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