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Cytokines and chemokines associated with Treg/Th17 response in chronic inflammatory periapical disease

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Abstract: Cytokines and chemokines have a fundamental role in the maintenance of inflammation and bone response, which culminate in the development of chronic periapical lesions. Regulatory (Treg) and Th17 cytokines play a key role in regulating the immune response involved in this process. The aim of this study was to investigate the role of Treg and Th17 cells in chronic inflammatory periapical disease, by comparing the expression of the immunoregulatory mediators TGF-β, IL-10, CCL4, and the proinflammatory IL-17 and CCL20 in the periapical tissue of teeth with pulp necrosis, with and without associated chronic lesions. Eighty-six periapical tissue samples were obtained from human teeth. The samples were divided into three groups: pulp necrosis with a periapical lesion (n=26); pulp necrosis without a periapical lesion (n=30), and control (n=30). All samples were submitted to histopathological analysis and cytokine and chemokine measurement through ELISA. Statistical analyses were done with Kruskal-Wallis and Mann-Whitney tests and Spearman correlation. The group with pulp necrosis and a periapical lesion showed a higher expression of CCL4 and TGF-β in comparison with pulp necrosis without a lesion. CCL20 was higher in the group with a periapical lesion when compared to the control. In all groups there was a weak positive correlation between IL-17/CCL20, IL-10/CCL4, and IL-17/TGF-β. Both types of cytokines, pro-inflammatory and immunoregulatory, occur simultaneously in periapical tissue. However, a rise in immunosuppressive cytokines and chemokines (CCL4 and TGF-β) in periapical lesions suggests a role of these cytokines in stable periapical disease.

Keywords: Cytokines; Endodontics; Periapical Diseases; T-lymphocytes, Regulatory; Th17 Cells.

Introduction

Inflammatory periapical disease is a consequence of the host's defense response to aggression originating from the root canal, appearing mainly as a sequel of infection and necrosis of the pulp caused by dental caries.[1] The immune response is fundamental in all stages of periapical disease. Bacteria and their metabolic products diffuse from the root canal and reach the dental apex stimulating first the innate immune-inflammatory response and then the adaptive immune response, which participate

in the maintenance and progression of periapical chronic lesions.[2]

A complex cytokine network acts in the development of periapical lesions resulting in a more complex scenario than that from responses of the pro and anti-inflammatory mediators Th1 and Th2.[3] Treg cells regulate Th1 and Th2 responses and play a central role in immunoregulation and induction of tolerance.[4] Cytokines like TGF-β and IL-10 control the proliferation and differentiation of naive T cells into Treg cells.[5,6] The traffic of T lymphocytes to inflamed sites is mainly regulated by cytokines and their receptors, and in this sense, CCL4 plays an important role in the immunoregulatory process, recruiting Treg cells into inflammatory sites.[7,8] The opposite role is exerted by Th17 cells, which produce the pro-inflammatory cytokine IL-17, among other mediators. IL-17 plays an important role in the induction of inflammation.[9] Data suggests that the up-regulation and stable expression of CCR6 is a fundamental feature for Th17 differentiation.[1]. CCR6 is expressed on subsets of T cells and dendritic cells and it has CCL20 as its unique ligand. CCL20 also acts as a chemoattractant recruiting Th17 cells.[11]

In respect to periapical inflammatory disease, the regulation of both Treg and Th17 cells has been mostly explored in animal models,[9,12] with only a few studies in humans.[2,13,14] Several cytokines and chemokines like TGF-β, IL-10, IL-17, CCL4, and CCL20 are involved in the immune responses that take place during the development of these lesions.[15] The presence of Treg cells was previously demonstrated in periapical lesions also expressing IL-10 and TGF-β and causing a suppressive effect in the immune response.[16] On the other hand, the production of IL-17 in periapical tissues was associated with an exacerbation of the inflammatory response, an elevated number of neutrophils, and bone resorption in human tissue.[17] The results of these studies highlight the antagonism between Treg and Th17 cells, however, they do not demonstrate if this balance is different between periapical tissues of teeth with necrotic pulp that developed chronic inflammatory lesions and those that did not.

Therefore, this study aimed to investigate the role of Treg and Th17 cells in chronic inflammatory periapical disease, comparing the expression of the immunoregulatory mediators TGF-β, IL-10, CCL4 and the proinflammatory IL-17 and CCL20 in the periapical tissue of teeth with pulp necrosis with and without associated chronic lesions. Likewise, we intended to compare the balance of these cytokines and chemokines in both situations with the sound tissue, contributing to the understanding of the dynamics of periapical disease. The hypothesis of this study was that periapical tissues of teeth with pulp necrosis and associated chronic lesions have different concentrations of cytokines and chemokines compared with necrotic pulp teeth without associated lesions.

Methodology

The study was approved by the Institutional Committee of Ethics (CAAE – 398983.14.1.0000.5149) and all participants signed an informed consent form. Teeth (n = 86) from patients who had an indication for a tooth extraction were prospectively collected at the Oral Surgery Clinic by a single trained person (ANOT). The teeth were assigned to three groups: Group 1, pulp necrosis with a periapical lesion (n = 26); Group 2, pulp necrosis without a periapical lesion (n = 30); and Group 3 (n=30, control) healthy included or semi-included third molars. The periapical tissue was obtained by curettage of teeth after extraction (group 1) or by scrapping the roots with a scalpel blade (group 2 and 3).

For the control group, periapical tissues were obtained from teeth extracted due to orthodontic indications and healthy third molars with no inflammatory process. In groups 1 and 2, all teeth had caries. Deciduous teeth were not included but no distinctions were made regarding the tooth type. In the case of multi-rooted teeth, only the tissue of the affected root was collected. Before extraction, teeth were examined and the pulp vitality was assessed by clinical examination, pulp vitality tests (thermal and electrical), and radiographic exam.[18] Teeth without pulp sensitivity and endodontic treatment indicated for extraction or endodontic apical surgery were included in the necrosis groups. Patients had no acute periapical symptoms at first appointment. Periapical lesions were characterized

radiographically as radiolucent lesions, a widened periodontal ligament space, and discontinuity of the lamina dura.[19] The diagnosis of periapical lesion was confirmed by histopathological exam.

The criteria for exclusion were patients with medical conditions requiring the use of systemic modifiers of bone metabolism (for example patients with mandibular osteonecrosis using bisphosphonates) or any other assisted drug therapy (systemic antibiotics, anti-inflammatory, or hormonal therapy) during the last 6 months before initiation of the study. Patients irradiated, with chronic periodontal disease, and pregnant or lactating women were excluded as well.

The periapical lesions were divided into two parts. One part was placed in 10% buffered formalin for routine processing and histopathological analysis. The other part was placed in 1.5 mL Eppendorf tubes and stored in a temperature range between -50°C to -86°C for cytokine dosage through ELISA assay.

Histopathological evaluation

The lesions samples (n = 26) were routinely processed and included in paraffin, then slides were stained with hematoxylin and eosin for microscopic evaluation of the samples. A diagnosis of a dental granuloma or a radicular cyst was established by a trained oral pathologist (MCFA). Dental granulomas are composed of granulation tissue, *i.e.*, a highly vascularized connective tissue interspersed by chronic inflammatory cells, without evidence of cavity formation or an epithelial lining. Radicular cysts are defined as cystic lesions lined by a non-keratinized stratified epithelium, which could (but may not) present hyperplasia, spongiosis, and exocytosis. The cyst capsule is of connective tissue with chronic inflammatory cells.

Detection of tissue cytokines and chemokines

The samples were weighed and homogenized in a buffer (0.4 mM NaCl, 10 mM NaPO, pH 7.4) containing protease inhibitors (0.1 mM phe- nylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, and 0.01 mg/mL aprotinin A) and polysorbate 20 (0.05%), pH 7.4, at a ratio of 1 mL solution per 100 mg tissue. The homogenate was centrifuged (8,946 g) at 4°C for 10 minutes. The supernatant was then collected and used for the quantification of chemokines. The levels of TGF-b, IL-17, IL-10, CCL20, and CCL4 were evaluated by ELISA, using commercially available kits (TGF-β and IL-10 from R&D Systems Europe Ltd., Abington, UK; IL-17, CCL4 and CCL20 from Abcam Plc Europe Ltd., Cambridge, UK). All assays were carried out according to the manufacturer's instructions. Samples were analyzed in duplicate. Concentration correction of cytokines and chemokines was done by the weight of each sample. This concentration was calculated according to the standard curve provided by the manufacturer of each kit used. The results were reported as picograms of cytokines or chemokines per 100 mg of tissue.

Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, version 22.0 (SPSS Inc., Chicago, USA). Normal distribution was tested using the Shapiro-Wilk procedure. Kruskal-Wallis and Mann-Whitney U tests were used for analyses of data with non-normal distributions. After applying the Bonferroni correction, statistical significance was considered when P values were < 0.017. Cytokine and chemokine levels were compared among the periapical lesion, pulp necrosis without a periapical lesion, and control groups. The Spearman correlation test was performed for the cytokines and chemokines IL-17/CCL20, IL-10/CCL4, and IL-17/TGF-β.

Results

The groups were composed of 26 periapical lesions (donors' age ranged from 19 to 51 years), 30 pulp necroses with no lesion (donors' age ranged from 22 to 58 years), and 30 controls (donors' age ranged from 18 to 27 years). Periapical lesions were composed of 19 dental granulomas (73.1%) and 7 radicular cysts (26.9%), but were not evaluated separately.

The levels of TGF-β, CCL4, and CCL20 were different among groups. TGF-β and CCL4 were higher in the periapical lesion group than in the necrosis/no lesion group (p=0.002 and p=0.001, respectively). TGF-β level was significantly lower

in the necrosis/no lesion group compared to the control group (p = 0.017), but the difference was not significant between the periapical lesion group and control group (p = 0.153). CCL4 and CCL20 had higher levels in the periapical lesion group than the control (p < 0.001), however their levels were not statistically different between group 2 and the control (p = 0.378 and p = 0.036 respectively). CCL20 levels were not different between the groups with pulp necrosis (p = 0.057). Regarding the IL-10 and IL-17 cytokines, there was no significant difference between the groups (Tables 1 and 2).

In all groups there was a weak positive correlation between IL-10/CCL4 (r = 0.285). The weak positive correlation observed between IL-17/CCL20 (r = 0.036) and IL-17/TGF-β (r = 0.021) was not statistically significant (Table 3).

Discussion

Th17 and Treg cells have opposite roles in the immune response process of inflammatory periapical disease. The principal cytokines and chemokines produced by those cells are TGF, IL-10, CCL4, CCL20, and IL-17. While Treg cells have a regulatory action mediated by TGF, IL-10, and CCL4,[2,7] the recruitment of Th17 cells is mediated via the interaction of their receptor, CCR6, with the chemokine CCL20,[20] which stimulates the local inflammatory cells to produce the cytokine IL-17.[5] This study explored a possible difference in this balance considering the presence or not of a periapical lesion, compared with controls.

TGF-β and CCL-4 levels were raised in the group with a periapical lesion compared to the group without a lesion. The increase in the expression of TGF-β in periapical lesions is possibly explained by the fact that cells producing this cytokine, such as eosinophils, lymphocytes, fibroblasts, and monocytes,[21] are found in high numbers in these lesions[22]. In addition, the increase of TGF-β1 in periapical lesions with more severe inflammation[2] may also be explained by an attempt to regulate the immunological mechanisms triggered in these lesions; thus, more advanced lesions imply a more complex regulatory response than early lesions.[13]

The increase of Treg regulators, especially TGF-β in chronic periapical lesions, is in accordance with other studies.[23,24] TGF-β can contribute to the stabilization of periapical lesions through its modulating effect on bone resorption activities mediated by the proinflammatory action of cytokines.[22]

Table 1. Descriptive analysis and comparison of the cytokine and chemokine levels among groups.

Cytokine/ chemokine	Groups	Mean	Std. deviation	p-value*
TGF-β	Control	482.20	511.54	
(pg/100 mg tissue)	Necrosis without lesion	274.58	582.64	
	Necrosis with lesion	921.31	911.22	0.004**
IL10	Control	208.51	438.41	
(pg/100 mg tissue)	Necrosis without lesion	6565.19	18152.98	
	Necrosis with lesion	709.18	1875.40	0.328
IL17	Control	76.10	51.37	
(pg/100 mg tissue)	Necrosis without lesion	75.53	76.42	
	Necrosis with lesion	79.52	60.55	0.633
CCL4	Control	121.69	152.59	
(pg/100 mg tissue)	Necrosis without lesion	151.85	157.03	
	Necrosis with lesion	657.82	704.24	< 0.001**
CCL20	Control	45.13	23.53	
(pg/100 mg tissue)	Necrosis without lesion	64.39	44.88	
	Necrosis with lesion	95.72	112.60	0.001**

*Kruskal Wallis Test. **Statistically significant (p < 0.05).

Table 2. Statistical comparison of cytokine and chemokine levels among groups.

Groups	Cytokine/ chemokine	p-value*
Control X	TGF-β	0.153
Necrosis with lesion	CCL4	< 0.001**
	CCL20	< 0.001**
Necrosis without lesion X Necrosis with lesion	TGF-β	0.002**
	CCL4	0.001**
	CCL20	0.057
Control X	TGF-β	0.017**
Necrosis without lesion	CCL4	0.378
	CCL20	0.036

* Mann-Whitney U Test. **Statistically significant ($p < 0.017$).

Table 3. Spearman correlation of the cytokines and chemokines IL-17/CCL20, IL-10/CCL4 and IL-17/ TGF-β.

Variables	Correlation coefficient (r)	p-value
IL-17 CCL20	0.036	0.768
IL-10 CCL4	0.285	0.050*
IL-17 TGF-β	0.021	0.875

*Statistically significant ($p \leq 0.05$).

In the present study, TGF-β, as well as other mediators, were also found in control tissues. This is not surprising, since this mediator is important in all physiological processes, contributing to the timely repair of injuries by the inhibition of bone resorption.[24] The control samples used in the present study were periapical tissues removed from a healthy tooth. Other studies opted to use dental pulp from impacted teeth as a control group.[17,25] Although our control tissue presented the advantage of being of the same nature as the diseased tissue, this might have allowed the inclusion of a very mild inflammatory process. Another interesting result was the increase in TGF-β expression in the control group compared to the group with necrosis and no lesion. This result might have been influenced by the inclusion of tissues from teeth extracted for orthodontic reasons in the control group. In this respect, it is known that TGF-β1 is a cytokine importantly associated with the process of bone remodeling that occurs during dental movement in orthodontic treatment.[26]

The roles of CCL20 and CCL4 in regulating the infiltration of Treg and CTL lymphocytes have already been demonstrated in other types of lesions,[27,28] but there are not studies on periapical disease. In the present study we found raised levels of CCL20 and CCL4 in periapical tissues of necrotic teeth. CCL20 is also involved in the recruitment of Th17 lymphocytes in chronic inflammatory processes of the liver,[29] triggering a pro-inflammatory response, for example, by the expression of the cytokine IL17. Considering this fact, it is interesting that we found a positive correlation between IL-17 and CCL20, similar to another study.[27] Although the correlation was weak, this observation may indicate that CCL20 recruits both Treg and Th17, lymphocytes that have opposite functions.[27]

Thus, the higher CCL20 expression in the periapical lesion group compared to the control group is implied with this dual function, suggesting that the triggering of a proinflammatory response, through the expression of the cytokine IL17, which at the same time is regulated via Treg cells and their mediators, contribute to the stability of those lesions. In fact, chronic periapical lesions can suffer a re-acutization process, which is correlated with an increase in leukocyte infiltration in the region, especially neutrophils, in addition to an increase of IL-17.[2,17] Studies have demonstrated an increase in IL17 expression in chronic periapical diseases undergoing re-acutization.[3,17]

Higher CCL4 levels were found in the periapical lesion group compared with the no lesion and control groups. This finding is interesting since this chemokine is related to the migration of CD8+ T lymphocytes,[27] responsible for important effector activities in the elimination of intracellular pathogens.[30] A study demonstrated that CCL4 was the most potent chemoattractant of Treg cells, suggesting that the recruitment of regulatory T cells by CCL4 plays a central role in the start of the humoral response.[7] Therefore, the fact that CCL4 is related to the migration of Treg lymphocytes,[31] which are capable of producing the immunosuppressive

cytokine IL-10,[32] might explain the positive correlation between IL-10 and CCL4 observed in our study. Thus, this immunosuppressive feature can represent a protective mechanism against bone destruction in the course of a periapical lesion.

Although weak, we found a positive correlation between IL-17 and TGF-β. These cytokines have an antagonist effect in periapical disease. While IL-17 causes exacerbation of inflammation in chronic periapical lesions,[33] TGF-β has immunosuppressive activities and promotes healing in the periapical region.[34] However, although TGF-β has an immunosuppressive action,[34] when it acts together with IL-6, it induces the differentiation of Th17 cells from naive precursors,[33] promotes the production of IL-17,[35] and in contrast, inhibits the development of Treg cells.[36] Thus, the combined action of TGF-β and IL-6 (produced by the innate immune system activated by infection) may "dictate" the balance between Treg and Th17 cells in the immune system.[36] Therefore, in the present study, the absence of IL-6 in the panel of analyzed cytokines was a limitation. Future research including the dosage of IL6 in an in vivo model similar to the present study is important to improve knowledge about controlling immune responses in periapical disease.

In this study, samples of radicular cysts and granulomas were not evaluated separately. Although some studies indicate a more inflamed profile for radicular cysts in comparison with granulomas,[24,25] our results are in accordance with others,[22,23] showing a predominance of an anti-inflammatory profile in chronic periapical disease. For future studies, the classification into either active or inactive lesions[3] rather than the histopathological type might be a more realistic analysis for chronic periapical disease.

In conclusion, this study showed the simultaneous immunosuppressive and pro-inflammatory features of chronic periapical lesions represented respectively by IL-10/TGF-β/CCL4 and IL-17/CCL20, with a predominance of Treg cytokines and chemokines, especially TGF-β. In all groups there was a weak positive correlation between IL-17/CCL20, IL-10/CCL4, and IL-17/TGF-β. These results suggest that the response of Treg/Th17 in chronic periapical lesions occurs not as a balance but as a co-stimulation mechanism. A large difference was not found between the pulp necrosis groups, however higher levels of Treg cytokines, especially TGF-β, were found in periapical lesions group than in necrotic teeth without a lesion, suggesting a role of this cytokine in the maintenance of periapical disease. Further research is needed on the dynamics of periapical inflammation and cytokine regulation network, employing a large panel of markers and focusing on the different stages of periapical disease.

Conclusion

The results of the present study showed that the Treg/Th17 profile in periapical tissues of necrotic teeth seems to be regulated by a co-stimulatory mechanism with pro-inflammatory and immunoregulatory mediators occurring simultaneously. In addition, the higher levels of immunoregulatory cytokines CCL4 and TGF-β in the presence of lesions probably contributes to their persistence.

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