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Differential expression of chemokines and chemokine receptors in inflammatory periapical diseases

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Background: Periapical lesions are thought to be the result of a local inflammatory response mediated by inflammatory cell infiltration and production of inflammatory mediators. Although chemokines are strongly implicated in the migration and activation of leukocytes in different inflammatory diseases and experimental models, little is known regarding the expression of chemokines and their receptors in human apical periodontitis.
Objective and methods: The objective of this study was to determine the expression of chemokines and their receptors by real-time polymerase chain reaction in samples obtained from healthy gingiva, periapical granulomas, and inflammatory periradicular cysts. The inflammatory infiltrate was characterized by immunohistochemistry.
Results: Comparing cysts and granulomas, an increase in $CD4^+$ and $CD8^+$ cells was observed in granulomas, despite the similar numbers of CD45RO-positive cells detected in both lesions. The analysis of mRNA expression revealed increased levels of CCR1, CCR2, CCR3, CCR5, CXCR1, and CXCR3 in both types of lesion compared with controls. Cysts exhibited a higher expression of CCR3, CCR5, CXCR1, and CXCR3 compared to granulomas. A significantly higher expression of RANTES, IP-10, and MCP-1 was detected in cysts compared with controls or granulomas. The expression of interleukin-8, MIP-1α, and MIP-1β was not different in the three experimental groups.
Conclusions: The increase in Th1 type (CCR1, CCR5, and CXCR3) and Th2 type (CCR2 and CCR3) receptors in both periapical lesions suggests the concomitant occurrence of Th1 and Th2 responses. Furthermore, the prevalent expression of the receptors CCR3, CCR5, CXCR1, and CXCR3 and of the chemokines RANTES, IP-10, and MCP-1 in cysts may point to a role in the progression of granulomas to cysts.

Key words: chemokines; periapical cyst; periapical granuloma

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Inflammatory periapical diseases are characterized by the persistent migration of polymorphonuclear leukocytes, monocytes, lymphocytes, plasma, and mast cells (14, 19, 21). Recently, there has been great interest in the mediators responsible for the selective recruitment and activation of these cell types in the periapical inflammatory sites. Of these mediators, interleukin (IL)-1α, IL-1β, tumor necrosis factor (TNF)-α, and chemokines have been the most thoroughly investigated, with their major cellular sources being macrophages and T lymphocytes, as well as stromal cells, such as fibroblasts and osteoblasts (8, 9, 17, 28). In the last decade, much attention has been focused on the chemokines, which are regarded as key mediators of leukocyte recruitment in different inflammatory models (18), including periapical lesions (3), and diseases (5). The chemokine family consists of small peptides known to mediate the recruitment of leukocytes and which are classified into four subfamilies according to the configuration of cysteine residues near the N-terminal, depending on whether the first two cysteines are separated (CXC) (CX3C) or not (CC) (C) by an intervening amino acid (22).

In inflamed periapical sites, as in other inflammatory diseases, two patterns of response can be generated: the Th1 type, characterized by the presence of IL-2, IL-12, TNF-α and interferon (IFN)-γ, and the Th2 type, where IL-4, IL-5, IL-6, IL-10, and IL-13 are present (7). Although the overall role of Th1 and Th2 responses in inflammatory periapical diseases has not been fully determined, the Th1 response appears to be predominant in granulomas (12) and the Th2 response in chronic lesions (8). Th1 and Th2 cells express different sets of chemokine receptors, which allow them to migrate to different tissues (2, 23–25). The receptors expressed preferentially on Th1 cells are CCR5, CXCR3 and, at lower levels, CCR1 (2, 10, 15, 16, 23, 24). The chemokine receptors CCR2 and CCR3 are expressed on Th2 cells, although CCR5 and CXCR3 can also be found at lower levels on this set of cells (2, 23, 24). CCR1, CCR2, and CCR5 are also expressed on monocytes and macrophages. Neutrophils were long thought to express only CXC chemokine receptors that were activated by ELR-containing CXC chemokines such as IL-8 (4, 20, 22). However, it was demonstrated recently that this cell type also expresses CC receptors (18).

The presence of IL-8, MIP-1-α, MIP-1β, IP-10, MCP-1, RANTES, and the receptors CCR5, CXCR3 and CCR3 has been previously reported in periapical granulomas (9, 17, 28). However, in those studies the expression of the chemokines and chemokine receptors was not analyzed by quantitative methods and cystic lesions were not systematically examined. Therefore, the chemokine pathways associated with inflammatory cell recruitment in the surrounding periapical tissues are poorly understood. The aim of the present study was to determine the levels of mRNA expression of the chemokines IL-8, MIP-1α, MIP-1β, RANTES, IP-10, and MCP-1 and of their receptors CCR1, CCR2, CCR3, CCR5, CXCR1, and CXCR3 in inflammatory periapical disease, using a quantitative method. In addition, we assessed the leukocyte subtypes present in the samples.

Material and methods

Human subjects

Periapical tissues were obtained under institutionally approved protocols and after informed consent had been obtained. Eighteen adult patients with radiographic evidence of periapical alveolar bone loss and indication of tooth removal were studied who had been referred to the Faculties of Dentistry of the University of São Paulo and of the University of Ribeirão Preto. The mean age of patients was 45 years (range 32–60 years). The patients had not taken any medication for the 2 months prior to the surgery and were apparently free of systemic diseases. Only two cases were endodontically treated. All cases were free of symptoms. The control group comprised three samples of clinically healthy gingiva taken during third molars removal. Cysts were diagnosed as presenting fully developed cavities lined by stratified squamous epithelium with variable thickness and a fibrous capsule. Periapical granulomas represented a severe infiltration of inflammatory cells with no epithelial lining. The samples were selected from a previous study of serial sections stained with hematoxylin and eosin.

Tissue preparation

The samples obtained from patients were divided in two equal parts. Half of each specimen was immersed in TRIZOL reagent (Gibco BRL, Life Technologies, Rockville, MD) and stored at −70°C. The second half was fixed in neutral buffered formalin, embedded by routine technique in paraffin wax and sectioned at 5 μm for hematoxylin and eosin and immunohistochemical staining.

Real-time polymerase chain reaction (PCR)

Complementary DNA (cDNA) was synthesized using 3 μg of RNA through a reverse transcription reaction (Superscript II, Gibco). Real-time PCR quantitative mRNA analyses were performed in an ABI Prism 5700 Sequence Detection System using the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK) for quantitation of amplicons. The standard PCR conditions were 95°C (10 min), and then 40 cycles of 94°C (1 min), 56°C (1 min), and 72°C (2 min), followed by the standard denaturation curve. The sequences of human primers were designed using PRIMER-EXPRESS software (Applied Biosystems) based on nucleotide sequences present in the GenBank database. The sequences of the primers, the predicted amplicon sizes, and the melting temperatures (Mt) used were as follows:

- β-actin sense ATGTTTGAGACCTTCAACAC, antisense CACGTCACACTTCATGATGG, which results in a 495- base pair (bp) amplification product;
- IL-8 sense GCAGCTCTGTGTGAAGGTGCAG, antisense CTGTTGTAGGGTTGCCAGATGC (366 bp, Mt 68°C).
- MIP-1α sense CTCTGCATCACTTGCTGCTGACAC, antisense GTCAGCGACCTGGAGCTGAGTG (212 bp, Mt 68°C);
- MIP-1β sense GCTAGTAGCTGCCTTCTGCTCTCC, antisense GGAGTACGTGTATGACCTGGAACTG (238 bp, Mt 68°C);
- IP-10 sense CCTGCTTCAAATATTTCCCT, antisense CCTTCCTGTATGTGTTTGGA (431 bp, Mt 66°C);
- RANTES sense TCATTGCTACTGCCCTCTGC, antisense CCCAGATTCTGACCACGACG (373 bp, Mt 70°C);
- MCP-1 sense AGGAAGATCTCAGTG CAGAGG, antisense AGTCTTCGGAGTTTGCCTTTG (177 bp, Mt 70°C);
- CCR1 sense CCTTCTGGATCGACTACAAGTT, antisense GTAGCAGATGATCATGACCAAC (396 bp, Mt 64°C);
- CCR2 sense GTAGGATTGCCCCACTCCAAA, antisense TACTGTTTTCCAACCCAGCTGG (302 bp, Mt 71°C);
- CCR3 sense CGAATTATGACCAACATCTACC, antisense GATAAATTCAGGAAGAGCTGCT (315 bp, Mt 64°C);
- CCR5 sense CTCTTCCTGCTCACACTACCAT, antisense TGTGTAGAAAATGAGGACTGCA (322 bp, Mt 72°C);
- CXCR1 sense TCTGCCTTTTGGGTCTTGTGAATA, antisense CCGGGCGTGGTGGTGAG (385 bp, Mt 71°C);
- CXCR3 sense TAGAAGTTGATGTTGAAGAGGG, antisense CAGCTCTTCCTATGACTATGG (339 bp, Mt 72°C).

PCR conditions for each target were conscientiously optimized with regard to primer concentration, absence of primer dimer formation, and efficiency of amplification of target genes and housekeeping gene control. SYBR Green PCR Master Mix (Applied Biosystems), 400 nM specific primers and 2.5 ng cDNA were used in each reaction. The threshold for positivity of real-time PCR was determined based on negative controls. The relative levels of gene expression were calculated according to the instructions in the User's Bulletin (P/N 4303859) from Applied Biosystems, by reference to the β-actin in the sample, using the cycle threshold (Ct) method. Briefly, Ct is the point at which the exponential increase in signal (fluorescence) crosses a somewhat arbitrary signal

level (usually 10 times background). The mean Ct values from duplicate measurements were used to calculate the expression of the target gene, with normalization to β-actin, and then compared with the target-internal control in control subjects to calculate the fold increase expression, using the 2–ΔCt formula. Negative controls without RNA and without reverse transcriptase were also performed. The results show one of three representative experiments.

Immunohistochemistry

From the tissues embedded in paraffin wax, 5-μm-thick sections were cut and collected on gelatin-coated glass slides. Immunohistochemical characterization of mononuclear cells was performed as previously described (21). Briefly, the samples were immersed in 3 mM citrate buffer (pH 6.0) for 10 min at 95°C for antigen retrieval. Subsequently, the sections were incubated in 3% normal goat serum (Vector Laboratories, Burlingame, CA) and then with one of the following antibodies: goat antihuman CD4 polyclonal antibody (C-18; Santa Cruz Biotechnology, Santa Cruz, CA); mouse antihuman CD68 monoclonal antibody (M0876; Dako, Glostrup, Denmark); mouse antihuman CD8 monoclonal antibody (M7103; Dako); mouse antihuman CD45RO monoclonal antibody (M0742; Dako), diluted in phosphate buffered saline (PBS) at 1 : 1000; 1 : 500; 1 : 200, and 1 : 500, respectively. The sections were then incubated with biotinylated goat antimouse or horse antigoat IgG antibodies (Vector Laboratories) followed by incubation with avidin-biotin complex (Vectastin ABC Kit, Vector Laboratories) and then in a solution of 3,3′-diaminobenzidine (0.7 mg/ml) (Sigma, St. Louis, MO). After washing, the slides were counterstained with Mayer's hematoxylin. Negative controls were obtained by the omission of primary antibodies. The number of positively stained cells for each antibody was counted per five consecutive microscopic high-power (× 100) fields. Results were expressed as the mean of positive cells and as the percentage of positive cells per field, calculated in relation to the total number of inflammatory mononuclear cells.

Statistical analysis

Data were analyzed using the Kruskal-Wallis test followed by Dunn's test and by multiple and simple regression analysis. *P*-values below 0.05 were considered significant.

Results

Thirteen lesions represented periapical granulomas characterized by a cell-rich granulation tissue, i.e. comprising lymphocytes, neutrophils, macrophages, fibrovascular elements, foam cells, Malassez epithelial islands, and Russel bodies, identified by their morphologic appearance. Five lesions represented periapical cysts characterized by a central fluid-filled, epithelium-lined cavity. The intensity of inflammatory infiltrate varied from moderate (six granulomas and three cysts) to intense (seven granulomas and two cysts). In 10 lesions (eight granulomas and two cysts) the infiltrate was predominantly mononuclear; in seven lesions (four granulomas and three cysts) the infiltrate was predominantly mononuclear and polymorphonuclear. In one case of granuloma, a polymorphonuclear infiltrate was predominant. Using hematoxylin and eosin stain, biofilms could be detected in two granuloma specimens. Epithelial odontogenic islands were found in eight samples, four of which were granulomas and four cysts.

In order to estimate phenotypic differences in the mononuclear inflammatory infiltrate, samples were analyzed by immunohistochemistry. The analysis of absolute and relative numbers of CD4, CD8, and CD68 positive cells revealed no predominance of any set of mononuclear cells within each periapical lesion (Table 1).

When comparing the two lesions, we observed an increase in absolute numbers of $CD4^+$ and $CD8^+$ lymphocytes in periapical granulomas. However, the population of T-memory $CD45RO^+$ and $CD68^+$ macrophages was similar in both types of lesions. The numbers of CD4 and CD8 positive cells in cysts were lower than in granulomas but the $CD45RO^+$ populations were similar in both lesions, which suggests that the majority of CD4 and CD8 lymphocytes in cysts are activated ($CD45RO^{low}$) (Table 1). No positive staining was observed when primary antibodies were omitted (data not shown).

The assessment of mRNA expression revealed significant levels of RANTES ($P = 0.017$), IP-10 ($P = 0.023$), and MCP-1 ($P = 0.049$) in cysts. The expression of these chemokines in granulomas was not significantly different from the control. Similar expression levels of IL-8, MIP-1α, and MIP-1β were observed in periapical lesions (cysts and granulomas) and control samples (Fig. 1). No expression of Mig was detected in any of the examined samples (data not shown). A significant increase in mRNA expression of CCR1, CCR2, CCR3, CCR5, CXCR1, and CXCR3 was observed in both types of lesion compared with controls. When comparing cysts and granulomas, mRNA expression of CCR3 ($P = 0.008$), CCR5 ($P = 0.030$), CXCR1 ($P = 0.033$), and CXCR3 ($P = 0.004$) were significantly higher in cysts (Fig. 1).

To examine the relationship between infiltrating cells, chemokines and chemokine receptors, multiple (MRA) and simple (SRA) regression analysis was performed. MRA of infiltrating cells vs. chemokines revealed a significant association of $CD4^+$ and $CD68^+$ with RANTES and of $CD8^+$ with MIP-1β and IP-10 in granulomas (Table 2). SRA confirmed the MRA data and also revealed an association between $CD8^+$ and MIP-1α and of $CD45RO^+$ with MIP-1α and MIP-1β in granulomas (Table 2). In cysts, SRA of infiltrating cells vs. chemokines did not indicate positive associations (Table 2). Moreover, MRA of infiltrating cells vs. chemokine receptors revealed an association between $CD8^+$ with CCR1 in granulomas and SRA showed an association of $CD4^+$ and $CD8^+$ with CCR2 in cysts (Table 2). Furthermore, a significant relationship was also observed between CCR1 and their ligands MIP-1α, MIP-1β, and RANTES in cysts (Table 2). When evaluating the correlation of microscopic findings, such as the presence of epithelial odontogenic islands and biofilms with chemokines and chemokine

Table 1. CD4, CD8, CD45RO, and CD68 positive cells (number and percentage) in periapical lesions

Cell marker	Control ($n = 3$)	Granuloma ($n = 13$)	Cyst ($n = 5$)
CD4	2.01 ± 0.48 (1.0)	19.89 ± 0.60[a,b] (17.1[a,b])	8.50 ± 1.50[a] (12.3[a])
CD8	2.40 ± 0.75 (2.5)	15.61 ± 1.20[a,b] (16.6[a])	9.23 ± 0.53[a] (15.6[a])
CD45RO	3.25 ± 0.85 (1.6)	13.81 ± 0.22[a] (15.3[a])	11.20 ± 1.01[a] (12.8[a])
CD68	4.50 ± 0.64 (6.0)	12.78 ± 0.74[a] (16.1[a,b])	10.8 ± 1.13[a] (12.76[a])

Results represent mean ± SEM of positive cells in five fields. Numbers in parentheses indicate the percentage of positive cells in relation of the total number of inflammatory mononuclear cells.
[a]Difference in relation to respective controls. [b]Difference between two lesion types.

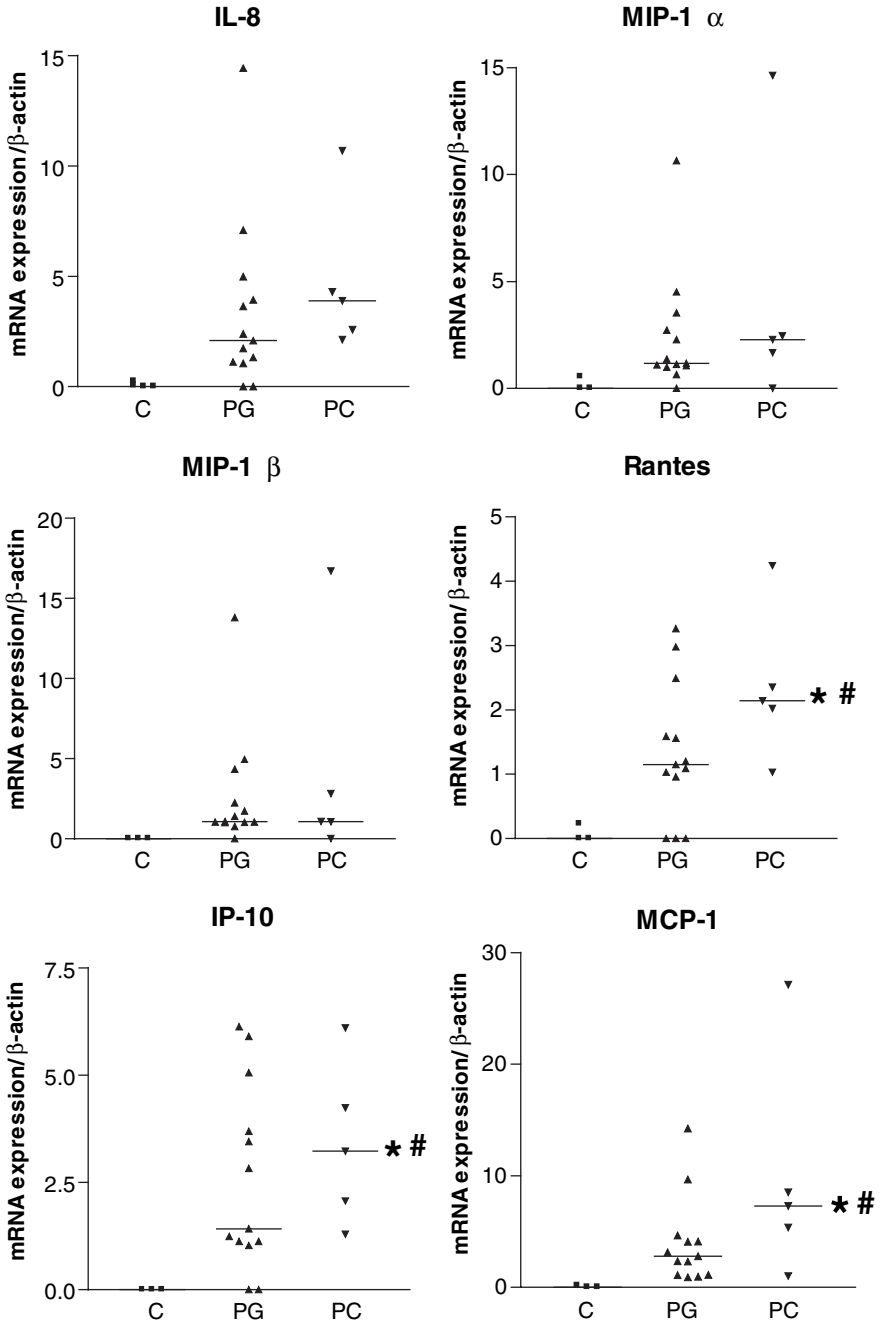

Fig. 1. Expression of chemokines and chemokine receptors in human inflammatory periapical lesions. Total RNA from healthy gingival tissues (C), periapical granulomas (PG) and cysts (PC) was extracted and the expression of IL-8, MIP-1α, MIP-1β, RANTES, IP-10, MCP-1, CCR1, CCR2, CCR3, CCR5, CXCR1, and CXCR3 was analyzed by real-time PCR. The mRNA expression was quantified as its ratio to β-actin. *$P < 0.05$ compared to controls. #$P < 0.05$ comparing two lesion types.

receptor expression, no positive significant relationship could be found (data not shown).

Discussion

Dental pulp is protected from microorganisms of the oral cavity by enamel and dentin. The exposure of dental pulp to microorganisms and their products, as a consequence of dental caries, fractures or operative procedures, triggers a pulp inflammatory response. The progression of pulp inflammation to periapex leads to innate and adaptive specific and nonspecific immune responses with periapical alveolar bone destruction and granuloma formation (19). Furthermore, apical granulomas can be converted to inflammatory cysts by poorly understood mechanisms. We examined whether a quantitative difference in chemokines and chemokine receptor expression is observed in different forms of apical periodontitis. We also investigated the cellular infiltrate present in these lesions.

Our results reveal a significant expression of the chemokine receptors CCR1, CCR2, CCR3, CCR5, CXCR1, and CXCR3 in both types of lesions. These

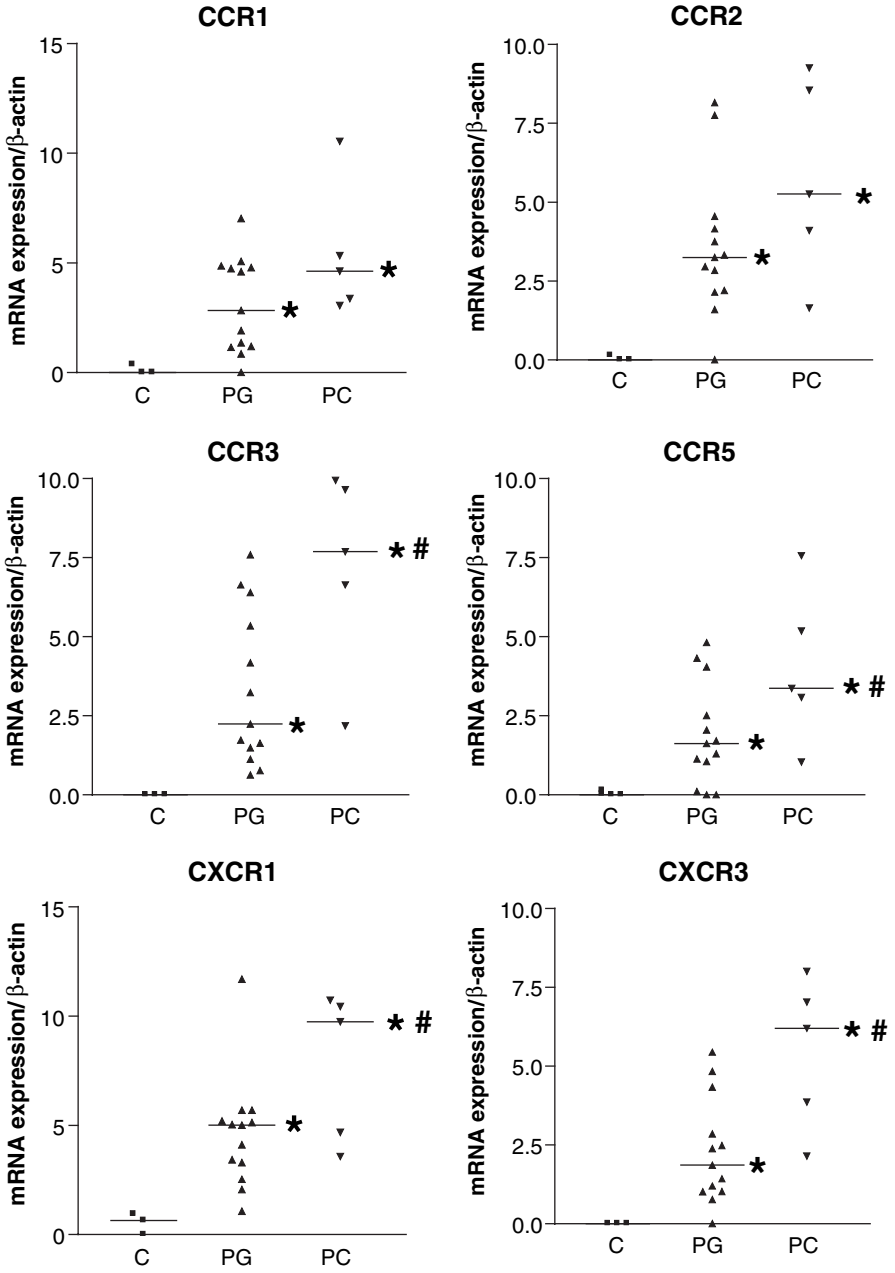

Fig. 1. Continued.

chemokine receptors are expressed on Th1 cells (CCR1, CCR5, and CXCR3), Th2 cells (CCR2 and CCR3), monocytes (CCR1, CCR2, and CCR5), and neutrophils (CXCR1). Consistently, the histopathologic analysis of lesions showed a significant infiltration of $CD4^+$ and $CD8^+$ lymphocytes, neutrophils, and macrophages. Although the distribution of Th1 and Th2 cells in periapical tissues was not investigated, we found a significant association of the $CD8^+$ population with CCR1 (a Th1 receptor) expression in granulomas. In cysts, on the other hand, $CD4^+$ and $CD8^+$ populations were found to be related to CCR2 (Th2 receptor).

In agreement with the findings in granulomas, previous studies also observed an increase in CCR5 and CXCR3 positive cells in this lesion type (9, 17, 28). Although the present study did not find a significantly different expression of IL-8, MIP-1α, MIP-1β, MCP-1, IP-10 or RANTES, previous studies showed an increase of these chemokines in periapical granulomas (9, 17, 28). These differences could be explained by the fact that we investigated the expression of the messenger RNA (mRNA), whereas other studies determined the expression of chemokines at the protein level. In addition, the above-mentioned studies employed immunohistochemistry, a nonquantitative approach, whereas we used real-time polymerase chain reaction, a quantitative method. In granulomas, the statistical regression analysis of chemokines vs. infiltrating cells may suggest that RANTES could participate in the recruitment of CD4 and CD68 positive cells, whereas MIP-1β, MIP-1-α, and IP-10 may be involved in $CD8^+$ recruitment. In addition, MIP-1β and

Table 2. Multiple and simple regression analysis of inflammatory cells, chemokines and chemokine receptors in periapical lesions

x y	MIP-1α	MIP-1β	RANTES	IP-10	MCP-1	CCR1	CCR2	CCR3	CCR5
Multiple regression analysis									
CD4	NS	NS	P=*0.066#* R^2A 0.260	NS	NS	NS	NS	NS	NS
CD8	NS	P=*0.024** R^2A=0.737	NS	P=*0.089#* R^2A=0.737	NS	P=*0.052#* R^2A=0.193	NS	NS	NS
CD45	NS	NS	NS	NS	NS	NS	NS	NS	NS
CD68	NS	NS	P=*0.038** R^2A=0.358	NS	NS	NS	NS	NS	NS

x y	MIP-1α	MIP-1β	RANTES	CCR2
Simple regression analysis				
CD4	NS	NS	[a]P=*0.049** *R^2=0.309*	[b]***P*=0.089#** **R^2=0.671**
CD8	P=*0.004** *R^2=0.539*	P=*0.001** *R^2 0.641*	NS	***P*=0.034*** **R^2=0.822**
CD45	P=*0.003** *R^2=0.571*	P=*0.0004** *R^2=0.698*	NS	NS
CD68	NS	NS	P=*0.008** *R^2=0.486*	NS
CCR1	***P*=0.012*** **R^2=0.908**	***P*=0.004*** **R^2=0.955**	***P*=0.023*** **R^2=0.862**	NS

None of the tested multiple comparisons involving more than two variables was available for cysts in function of the number of samples. Simple regression analysis was performed comparing two variables.
[a]*Italic*: granulomas. [b]**bold:** cysts.
NS: nonsignificant for granulomas and cysts. R^2: percentage of variation of variable y that is explained by variable x. R^2A: adjusted R^2 was used for multiple analysis. **P*-values significant at a level of 5%. #*P*-values significant at a level of 10%.

MIP-1-α were related to $CD45RO^+$ cell infiltration. However, we cannot exclude the possibility that these cell types might also be active as a source for each respective chemokine expression in periapical tissues. Moreover, different cell sources, such as neutrophils, fibroblasts, and osteoblasts, may account for chemokine production in inflammatory periapical diseases.

In cystic lesions, parallel with the increase in mRNA expression of the chemokine receptors, an increase was also detected in the ligands RANTES (CCR1, CCR3, CCR5), and IP-10 (CXCR3), which are associated with the Th1 response, and MCP-1 (CCR2), which has been associated with Th2 responses (2, 4, 6, 10, 11, 15, 16, 23, 24). Moreover, simple regression analysis indicated a significant association of CCR1 with the ligands MIP-1α, MIP-1β, and RANTES in cysts. Together, the results suggest that Th1 and Th2 responses are present concomitantly in cystic lesions. Furthermore, RANTES (29) and MCP-1 (30) expression has been implicated in the recruitment of osteoclast precursors and may contribute to the maintenance of resorptive activity in sites of periapical bone resorption. The greater expression of the chemokines (RANTES, IP-10, and MCP-1) and chemokine receptors (CCR3, CCR5, CXCR1, and CXCR3) in cysts compared with granulomas may have some importance in the evolution of granulomas to cysts. In fact, chemokines may be implicated in the proliferation of Malassez epithelial rests, from which the cyst lining originates (17).

As discussed above, our results concerning mRNA expression of chemokine receptors associated with Th1 as well as Th2 responses suggest that these two patterns are concomitant in both cysts and granulomas. This is supported by studies indicating that the Th1 and Th2 patterns are concomitant in periapical lesions (12). However, there is also evidence that the Th2 pattern prevails in chronic lesions (8). Moreover, results obtained from experimental models have suggested a hierarchy of Th2 cytokines in the immunomodulation of apical periodontitis, given that in the absence of Th1-type cytokines IFN-γ and IL-12, the formation of periapical lesions was not modified (26), whereas mice deficient in IL-6 (1) and IL-10 (27) Th2 cytokines showed a significant increase in the extent of apical lesions. This apparent discrepancy could be a consequence of differences in the age of the pathologic process and/or microbial infections. In this regard, it is noteworthy that the endodontic pathogenic microorganisms *Porphyromonas endodontalis*, *Porphyromonas gingivalis,* and *Prevotella intermedia* are able to induce the production of IL-8 by pulp fibroblasts and osteoblasts (31) and the production of MIP-1α and MIP-1β by stimulated neutrophils (13).

In conclusion, our results show that chemokine receptors and their ligands are differentially expressed during periapical inflammatory processes, suggesting that they may be implicated in the progression of apical periodontitis and, consequently, lead to difficulties in achieving resolution even after endodontic therapy. Chemokines may represent attractive targets for immunomodulation of apical periodontitis.

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