International Endodontic Journal

doi:10.1111/j.1365-2591.2010.01764.x

Monocyte chemotactic protein-3: possible involvement in apical periodontitis chemotaxis

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Abstract

Dezerega A, Osorio C, Mardones J, Mundi V, Dutzan N, Franco M, Gamonal J, Oyarzún A, Overall CM, Hernández M. Monocyte chemotactic protein-3: possible involvement in apical periodontitis chemotaxis. *International Endodontic Journal*, **43**, 902–908, 2010.

Aim To study the expression of monocyte chemotactic protein-3 (MCP-3, also known as chemokine CCL-7) in tissue from apical lesions (AL) and to associate MCP-3 expression with symptomatic or asymptomatic apical periodontitis.

Methodology To determine the expression of MCP-3 in AL, biopsies obtained during tooth extraction procedures were fixed, subjected to routine processing and diagnosed as apical granuloma (AG) (n = 7) or radicular cyst (RC) (n = 5). As controls, apical periodontal ligament (PDL) specimens from healthy premolars extracted for orthodontics reasons were included (n = 7). All specimens were immunostained for MCP-3 and examined under a light microscope. In addition, homogenates from AL (n = 14) and healthy PDL samples (n = 7) were studied through immunowestern blot. Finally, periapical exudates samples were collected from root canals of teeth having diagnosis of symptomatic (n = 14) and asymptomatic apical periodontitis (n = 14) during routine endodontic treatments and analysed by immunowestern blot and densitometry.

Results MCP-3 was detected in AG and RC and localized mainly to inflammatory leucocytes, whereas no expression was observed in healthy PDLs. MCP-3 was also detected in periapical exudate, and its levels were significantly higher in symptomatic than in asymptomatic apical periodontitis.

Conclusions MCP-3 was expressed in AL and its levels associated with clinical symptoms. MCP-3 might play a role in disease pathogenesis, possibly by stimulating mononuclear chemotaxis.

Keywords: Apical periodontitis, chemokines, MCP-3.

Received 11 December 2009; accepted 1 May 2010

Introduction

Apical periodontitis develops as a response to root canal infection and the presence of apical bone resorption represents the radiographic hallmark of these lesions (Ataoglu *et al.* 2002). Histologically, apical periodontitis is referred to as AG and can evolve to form a radicular cyst (RC). AG consists of inflamed granulation tissue circumscribed by a fibrous capsule, whilst a RC is characterized by the formation of an epithelium lined cavity (Nair 2004). Until now, a large number of cells, cytokines and enzymes involved in these lesions have been described; however, the molecular mechanisms underlying this pathogenesis are not fully understood.

The presence of mononuclear immunocompetent cells such as macrophages, T and B cells and plasma cells has been demonstrated in human periapical lesions, suggesting that the host immune response is

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involved in the pathogenesis of the disease (Ataoglu *et al.* 2002). Amongst them, macrophages have been considered the major constituents of apical lesions (AL) (Metzger 2000). Their roles can be broadly divided into those with trophic effects, such as the production of enzymes, cytokines and chemokines, and those related to phagocytosis of foreign material or apoptotic cells (Pixley & Stanley 2004). Osteoclasts, which are also derived from mononuclear phagocytic precursors, resorb bone matrix under physiological and pathological conditions, playing a pivotal role during apical periodontitis bone resorption (Pixley & Stanley 2004). The arrival of leucocytes and osteoclast precursor cells obeys to the expression of chemoattractant proteins at the periapex, contributing to apical periodontitis development and progression. Chemotaxis corresponds to recruitment and migration of specific cell populations in response to the generation of chemotactic gradients by locally produced and secreted chemokines. Chemokines are a complex superfamily of mediators mainly involved in immune and inflammatory responses (Combadiere *et al.* 1995). Besides the ability to recruit leucocytes directly by providing a chemotactic gradient, chemokines can also activate integrins, stimulate the release of inflammatory mediators and modulate vascularization, thereby exacerbating inflammatory process (Mackay 2001). Structurally, chemokines are characterized by four conserved Cys residues, and based on its relative position, Cys, Cys-X-Cys, Cys-X3-Cys (CXC) and Cys-Cys (CC) families are distinguished. CXC chemokines act primarily on neutrophil leucocytes during the initial phases of inflammation, whereas CC chemokines stimulate monocytes, basophil and eosinophil leucocytes, as well as lymphocytes (Combadiere *et al.* 1995). The monocyte chemotactic proteins (MCPs) of the CC family consists of five proteins termed MCP-1, MCP-2, MCP-3, MCP-4 and MCP-5 that target multiple leucocytes subsets, mainly monocytes and lymphocytes (McQuibban *et al.* 2002).

Although MCP-3 is one of the most pluripotent chemokines, it acts predominantly on monocyte-macrophage lineage (Polentarutti *et al.* 1997) by joining its receptors CCR-1, CCR-2, CCR-3 and CCR-5. MCP-3 is expressed by endothelial cells, monocytes and fibroblasts induced by IL-1β, TNF-α, IFN-γ and lipopolysaccharide (LPS) and thereby it is highly expressed in chronic inflammatory disorders, such as rheumatoid arthritis (Haringman *et al.* 2006) and chronic periodontitis (Dezerega *et al.* 2010) both of them characterized by progressive bone resorption. Whereas expression of MCP-1 has previously been documented in apical periodontitis (Kabashima *et al.* 2001), the expression of MCP-3 in these lesions remains unknown. The aim of this study was first to determine whether MCP-3 is present in AL; and second, to address whether MCP-3 expression is associated with symptomatic apical periodontitis.

Materials and methods

Patients

A total of 26 adult patients with a diagnosis of apical periodontitis and indication for tooth extraction, and 14 periodontally healthy subjects having an indication of tooth extraction for orthodontic reasons and complete root development were selected from the Clinics of Diagnostic and Surgery, School of Dentistry, University of Chile, Santiago, Chile. The diagnosis of apical periodontitis was based on clinical and radiographic examination. Clinically, the patients had reported no symptoms, spontaneous pain nor positive response to percussion; involved teeth had to be non-responsive to pulp sensibility tests. Radiographically, radiolucent AL were identified as clear bone loss and disappearance of the periodontal ligament (PDL) space at the tooth apex. Another group of patients from the Clinic of Endodontics, from the Faculty of Dentistry, University of Chile, having symptomatic ($N = 14$) and asymptomatic ($N = 14$) apical periodontitis were also selected. Symptomatic apical periodontitis was defined based on the following criteria: clinical and radiographic examination that determined the existence of periradicular pathosis involving destruction of cortical bone and painful sensitivity to percussion and/or palpation, whereas asymptomatic apical periodontitis was defined based on the same radiographic criteria, but manifesting slight or no sensitivity to percussion (Radics *et al.* 2003). Exclusion criteria included history of systemic disorder such as diabetes mellitus, osteoporosis, or patients who had received antibiotic, anti-inflammatory, or hormonal drugs within the past 3 months period prior to the study. The protocol was clearly explained to all patients, and Institutional Reviews Board-approved informed consents were signed.

AL biopsies and immunohistochemistry

Biopsies from 12 AL and seven healthy apical PDLs were obtained during tooth extraction, fixed in 10% buffered formalin and embedded in paraffin. Sections of 6 μm thick were cut and mounted on glass slides, routinely

processed and stained with haematoxylin–eosin for histological examination. RCs (n = 5) were diagnosed as presenting fully developed cavities lined by stratified squamous epithelium with variable thickness and a fibrous capsule. AG (n = 7) was included if granulation tissue without epithelium was found; and healthy apical PDL had no signs of inflammation. To determine MCP-3 expression, immunohistochemistry was conducted. Briefly, specimens were decalcified with 12.5% EDTA for processing whole healthy premolars, and 6-μm-thick sections were mounted onto silane-coated glass slides, deparaffinized and dehydrated. Sections were then immersed in 3% methanol–hydrogen peroxide solution to block endogenous peroxidase activity, followed by blocking with horse normal serum, incubation with monoclonal primary mouse anti-human MCP-3 antibody overnight at 4 °C (Abcam®, Cambridge, UK), 1 : 100 dilution. After being washed in phosphate buffered saline (PBS) (Hemagen® Diagnostics Inc., Columbia, KS, USA), the sections were incubated with anti-mouse biotinylated secondary antibody and avidin–biotine–peroxidase complex (Kit ABC Universal, RTU Vectastain® kit, Burlingame, CA, USA); then the reaction was developed with DAB (Peroxidase Sustrate Kit DAB SK4100; Zymed Labs, San Francisco, CA, USA) and counterstained with haematoxylin. For negative controls, the sections were incubated similarly, but without adding the primary antibody.

Periapical tissue samples and homogenates

A total of fourteen AL and seven healthy apical PDLs were obtained during tooth extraction and kept at −80 °C until tissue homogenization. The homogenization was performed with NaCl 0.9% with proteases inhibitor cocktail (Complete, mini, EDTA-free; Roche, Indianapolis, IN, USA). Total protein content was quantified using bicinchoninic acid (BCA) assay (Micro BCA; Pierce, Rockford, IL, USA) according the manufacturer's instructions and analysed by Western blotting and densitometry.

Periapical exudates collection and elution

Periapical exudate samples were collected from symptomatic (n = 14) and asymptomatic (n = 14) apical periodontitis patients during root canal treatment, as previously described (Shimauchi *et al.* 1996). Briefly, during the first session of each root canal treatment, after the access cavity was finished and prior to the use of any canal irrigant, a sterile paper point size 25 (Dentsply Maillefer, Ballaigues, Switzerland) was inserted into the canal and left in place for 30 s. The paper point was maintained in Eppendorf tubes at −80 °C until elution. The elution was performed with 100 μL of elution buffer for 30 min at 4 °C (Tris HCl 50 mmol L^{-1}, TRITON X-100 0.01% and NaCl 0.2 mol L^{-1}) per paper point and centrifuged at 4 °C and 18 000 g for 5 min. For quantification of total protein content, BCA assay (Micro BCA; Pierce) was conducted according the manufacturer's instructions. Then, aliquots of each eluate were used for Western blot assays.

Western blot analysis

To analyse MCP3 immunoreactivities, aliquots of periapical exudates and homogenates were loaded into 20% Tris-Tricine SDS-PAGE (Laemmli 1970) and transferred to a polyvinylidene fluoride (PVDF) membranes. As positive control, human recombinant MCP-3 was used. For western hybridization, the membrane was blocked with 3% skim milk in Tris-buffered saline – Tween 0.1% (TBS-T) for 1 h. Primary antibody against MCP-3 (R&D Systems, Minneapolis, MN, USA) diluted 1 : 250 in 3% skim milk TBS-T was added, and the membrane was incubated overnight. Then, the membrane was washed with TBS-T, incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody, washed with TBS-T again, and hybridized bands were detected with an enhanced chemiluminiscence detection kit (Femto; Pierce). Gels were digitized and integrated density of immunoreactive bands was calculated using the UN-SCAN-IT gel automated digitizing system V4.1 software (Silk Scientific Corporation, Orem, UT, USA). Results were expressed as arbitrary units of density (pixels)/μg of total protein content. To assess differences between symptomatic and asymptomatic apical periodontitis, unpaired t-tests were conducted, using Stata V10 software.

Results

Analysis of MCP-3 expression showed positive immunostaining in all examined AG and RCs and none of the healthy apical PDL samples (Fig. 1 A–F). MCP-3 was localized mainly to vascular endothelial cells and inflammatory infiltrate from AG, as well as the capsule of RC; the most prominent cells expressing the chemokine corresponded to plasma cells and lymphocytes. A faint MCP-3 immunoreactivity was also detected in the lining epithelium from RCs.

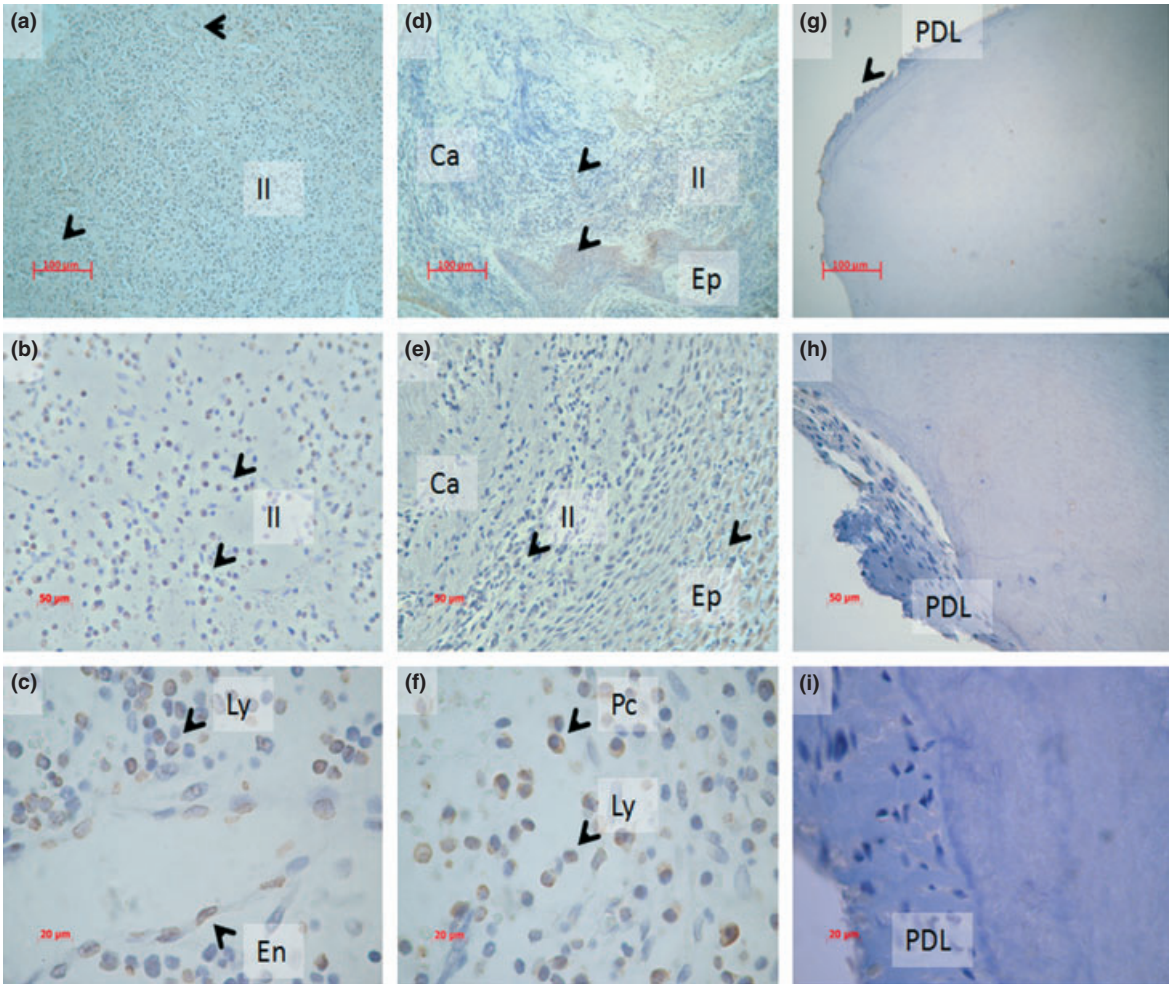

Figure 1 Monocyte chemotactic protein-3 expression in apical lesions and healthy periodontal ligament (PDL). A–C, apical granuloma; D–F, radicular cyst; G–I: Healthy PDL. Arrowheads show immunostained cells. II, Inflammatory infiltrate; Ep, Epithelial cyst lining; Ca, Cyst capsule; PDL, Periodontal ligament; Ly, Lymphocytes; En, Vascular endothelial cells and Pc, Plasma cells.

MCP-3 immunoreactivities in AL and healthy apical PDLs homogenates are shown in Fig. 2. In AL samples, MCP-3 was identified as a unique 13-kDa band. No expression of MCP-3 was detected in healthy apical PDL samples. In periapical exudates from diseased subjects, similar immunoreactivities were identified. When comparing symptomatic and asymptomatic apical periodontitis using densitometric analysis, MCP-3 levels were significantly higher in the former group (Table 1).

Discussion

One of the key phenomena of apical periodontitis development is chemotaxis. The results demonstrate for the first time the presence of MCP-3 in AL biopsies and periapical exudates from apical periodontitis affected teeth. MCP-3 corresponds to a highly potent and pleiotropic chemokine involved mainly in leucocyte chemotaxis, and its expression has been demonstrated to be elevated in a number of different chronic inflammatory diseases characterized by bone destruction, including rheumatoid arthritis (Haringman *et al.* 2006) and chronic periodontitis (Dezerega *et al.* 2010). In this study, we demonstrated through immunohistochemistry that AG and RCs from apical periodontitis patients express MCP-3, and we confirmed the presence of MCP-3 in AL homogenates by Western blot. Conversely, MCP-3 was not detected in healthy PDL

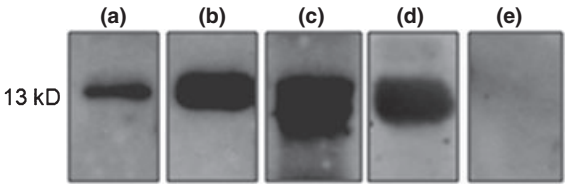

Figure 2 Monocyte chemotactic protein-3 (MCP-3) immunoreactivities in apical biopsies homogenates and periapical exudates from symptomatic and asymptomatic apical periodontitis. A: MCP-3 positive control; B: PE exudate from asymptomatic apical periodontitis; C: periapical exudate from symptomatic apical periodontitis; D: apical lesions homogenate; E: Healthy periodontal ligament homogenate.

samples. Furthermore, we observed a direct association amongst MCP-3 expression levels and clinically symptomatic apical periodontitis. The strong MCP-3 expression in AL might be explained by local upregulation of the chemokine within periapical inflammatory focus. Stimulation by proinflammatory mediators such as IL-1β, TNF-α and LPS can induce MCP-3 expression (Menten *et al.* 2001), and their presence has been well documented in these lesions (Ataoglu *et al.* 2002, Hong *et al.* 2004).

MCP-3 is produced mostly by monocytes, endothelial cells and fibroblasts and acts by joining its receptors CCR-1, CCR-2, CCR-3 and CCR-5 present in a broad spectrum of leucocytes (Menten *et al.* 2001). In AG and RC, MCP-3 presence was associated mainly with inflammatory infiltrate and vascular endothelial cells. Within the inflammatory cells, lymphocytes and plasma cells showed the strongest immunoreactivity (Vernal *et al.* 2006). Accordingly, in experimentally induced periapical lesions in rats at all time-points, lymphocytes were found to be the predominant cell type (Yu & Stashenko 1987), whereas plasma cells were detectable at 14 days and increased their presence with time (Kawashima *et al.* 1996). Hence, the latter two cell types could act as continuous MCP-3 sources because of the very initial phases of AL development. On the other hand, MCP-3 immunoreactivity might also originate from target cells expressing MCP-3/CCR complex on their surface. Accordingly, CCR expression has been well documented in AL (Kabashima *et al.* 2001, Silva *et al.* 2007). Furthermore, MCP-3 was also expressed in vascular endothelium, confirming its potential role in leucocyte recruitment from blood vessel circulation to diseased periapical tissue.

Table 1 Densitometric analysis of monocyte chemotactic protein-3 immunoreactivities from periapical exudates in symptomatic and asymptomatic apical periodontitis patients

Asymptomatic apical periodontitis		Symptomatic apical periodontitis	
Patient	Levels (au/μg)	Patient	Levels (au/μg)
1	182 951	1	306 354
2	25 839	2	146 005
3	149 735	3	407 656
4	104 134	4	332 603
5	68 456	5	149 114
6	60 042	6	161 345
7	68 781	7	184 067
8	106 981	8	150 355
9	66 698	9	206 267
10	112 539	10	132 732
11	71 442	11	211 945
12	210 678	12	111 793
13	102 379	13	270 395
14	212 800	14	15 363
Mean ± SD	110 247 ± 58 471*	Mean ± SD	198 999 ± 101 244*

au, arbitrary units of density (pixels).
*$P < 0.05$ (unpaired *T*-test).

Amongst leucocytes, MCP-3 targets mainly cells from monocyte–macrophage linage, from which macrophages and osteoclasts are derived (Menten *et al.* 2001). In AL, macrophages are considered to have a major role because they produce a broad spectrum of inflammatory mediators including IL-1β, TNF-α and RANKL, as well as effector molecules, such as enzymes and prostaglandins, and participate in phagocytosis during immune response (Metzger 2000, Vernal *et al.* 2006). RANKL is one of the main cytokines involved in bone resorption, because it takes part in osteoclast differentiation and activation. To reach the periapical area, preosteoclasts and osteoclasts must migrate from circulation and surrounding tissues obeying to chemotactic gradients. It is well documented that MCP-3 is capable to attract preosteoclasts and osteoclasts to the sites of bone resorption (Yu *et al.* 2004). Consequently, MCP-3 detection in AL could explain at least partially the presence of osteoclasts and macrophages in AL.

In this study, MCP-3 was also detected in periapical exudates from apical periodontitis teeth. Periapical exudates taken from root canals contain serum plasma-derived components, as well as locally produced inflammatory mediators reflecting local and systemic immune responses elicited from apical periodontitis (Shimauchi *et al.* 1996). In plasma from healthy subjects, MCP-3 is almost undetectable (Yanaba *et al.* 2006) and similarly occurs in gingival crevicular fluid (GCF) from healthy subjects. GCF corresponds to plasma exudate, containing mediators involved in

tissue inflammation and destruction during gingivitis and periodontitis. GCF MCP-3 expression has previously been reported during chronic periodontitis, and its levels raises significantly during the progression of the disease, but no MCP-3 expression was observed in GCF from healthy subjects (Dezerega *et al.* 2010).

MCP-3 expression levels were significantly higher in periapical exudates from symptomatic apical periodontitis than in asymptomatic cases. Similarly, previous reports have demonstrated that the levels of certain inflammatory mediators such as IL-1α, IL-6 and PGE_2 are elevated in symptomatic compared to asymptomatic apical periodontitis (Takayama *et al.* 1996, Shimauchi *et al.* 1998, Radics *et al.* 2003). IL-6 and PGE_2 are able to induce IL-1α expression, and IL-1α in turn stimulates MCP-3 synthesis and release, which could explain the higher MCP-3 expression found in symptomatic apical periodontitis. Although neutrophils have been considered the main cell population during acute apical periodontitis according to some authors (Colic *et al.* 2009), other studies suggest an active role for macrophages during acute exacerbation of apical periodontitis (Baqui *et al.* 1998). The present results suggest that MCP-3 is related to symptomatic apical periodontitis, probably by contributing to monocyte–macrophage chemotaxis and recruitment to apical tissues.

Conclusion

This study provides new and interesting findings on the presence of MCP-3 in AL and apical periodontitis exudates. Taken together, the results strongly support that MCP-3 is involved in apical periodontitis pathogenesis.

Acknowledgements

This study was supported by DI 07/02-2 and FONDECYT 1090461 funding. The authors express their gratitude to Leslie Henríquez for her excellent technical assistance, to Consuelo Fresno for her collaboration in sample collection, and to the Endodontic Society of Chile for its continuous support.

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