Expression and immunohistochemical localization of leptin receptor in human periapical granuloma

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Abstract


Aim To investigate the expression and immunohistochemical localization of leptin receptor (LEPR) in human periapical granulomas.

Methodology Periapical inflammatory lesions were obtained from extracted human teeth and teeth which underwent periapical surgery. After their histopathological categorization as periapical granulomas (n = 20), they were examined by immunohistochemistry using human LEPR monoclonal antibodies. LEPR mRNA expression was also determined by quantitative real-time PCR (qRT-PCR), and the amount of LEPR protein was analysed by immunoblot.

Results All granuloma samples expressed LEPR. Amongst inflammatory cells, only macrophages showed expression of LEPR. Western blot analysis revealed the presence in the samples of a protein with apparent molecular weight of ~120 kDa, corresponding to the estimated molecular weight of LEPR. The qRT-PCR analysis demonstrated the expression of LEPR mRNA, corresponding the size of the amplified fragment (338 bp), assessed by agarose gel electrophoresis, to that of LEPR mRNA.

Conclusions Human periapical granulomas express LEPR. In periapical granulomas, only macrophages showed expression of LEPR. This finding suggests that leptin can play a role in inflammatory and immune periapical responses.

Keywords: apical granuloma, immune system, immunohistochemistry, leptin, leptin receptor, periapical inflammatory response.

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Introduction

Leptin is a 16 kDa nonglycosylated polypeptide hormone of 146 amino acids encoded by the Ob gene (Zhang et al. 1994). Leptin was initially described as an adipocyte-derived hormone to regulate weight control (Flier 1995), but it has also been classified as a pro-inflammatory cytokine (Zhang et al. 1994, Sánchez-Margalet et al. 2003). A role for leptin regulating immunity, inflammation and haematopoiesis has been accepted (Cioffi et al. 1996, Fantuzzi & Faggioni 2000, Sánchez-Margalet et al. 2003). Consistent with this role of leptin in the mechanisms of immune response and host defence, leptin levels are increased upon infectious and inflammatory stimuli such as LPS, turpentine and cytokines (Matarese 2000). Accordingly, leptin receptor (LEPR) shows sequence homology to members of the class I cytokine receptor superfamily (Tartaglia et al. 1995), such as the IL-6-type cytokine receptors, leucocyte inhibitory factor and granulocyte colony-stimulating factor.
LEPR in periapical granuloma  Martín-González et al.

(Baumann et al. 1996). LEPR is expressed not only in the central nervous system, but also in haematopoietic and immune systems (Sánchez-Margalet et al. 2003), in mice monocytes and lymphocytes (Loffreda et al. 1998, Lord et al. 1998) and in human peripheral blood T lymphocytes (both CD4 and CD8; Martín-Romero et al. 2000, Sánchez-Margalet et al. 2003).

In relation to oral tissues, LEPR immunoreactivity has been found in the gingival epithelium (Ay et al. 2012). LEPR gene has been detected in experimental rats periapical lesions (Martínez et al. 2007), and both LEPR protein and LEPR mRNA have been described in healthy and inflamed human dental pulp (Martín-González et al. 2013a). The upregulation of LEPR (Martín-González et al. 2013a) and leptin (Martín-González et al. 2013b) expression in inflamed human pulp samples suggests that leptin can play a role in inflammatory and local immune responses in human dental pulp.

Chronic periapical lesions result from a localized inflammatory reaction to the bacteria and by-products of the root canal system. The most common periapical lesions are periapical granulomas (Gbolahan et al. 2008). Periapical granuloma is a chronic inflammatory lesion at the apex of a tooth with a nonvital pulp consisting of granulation tissue and scar. The inflammatory cell infiltrate in these chronic periapical lesions consists of a mix of plasma cells, T and B lymphocytes, macrophages, polynuclear leucocytes (PMNs), dendritic cells (DCs), natural killer cells (NK cells) and mast cells, present in different proportions within the granulation tissue of periapical lesions (Márton & Kiss 1993, 2014, Stashenko et al. 1998, Liapatas et al. 2003). The inflammatory infiltrate constitutes approximately 50% of the cells present in periapical granulomas, with noninflammatory connective tissue cells, including fibroblasts, vascular endothelium, proliferating epithelium, osteoblasts and osteoclasts comprising the balance (Langeland et al. 1977, Yu & Stashenko 1987).

The presence of leptin has been demonstrated in chronic periapical lesions (Haghighi et al. 2010) suggesting that leptin could play a role in the inflammatory and immune responses in periapical tissues. However, the expression of LEPR by inflammatory cells present in periapical granulomas has not been studied.

The aim of this study was to investigate the expression of LEPR in human periapical granulomas. Immunohistochemistry was used to determine whether LEPR is expressed on the surface of infiltrating cells.

Materials and methods

The study was carried out with the understanding and written consent of each subject and according to the principles of the World Medical Association Declaration of Helsinki. The protocol was approved by the Ethical Board of the University.

Human chronic periapical lesions samples

Twenty human chronic periapical lesions from twenty healthy, nonsmoking, human donors (45–72 years old), who gave their written informed consent, were obtained from 14 freshly extracted teeth and six teeth which had undergone periapical surgery. Inflammatory tissues surrounding the periapical areas were dissected. Each sample was then divided into three parts, one for the Western blotting analysis, another for RNA extraction and quantitative real-time PCR (qRT-PCR) assay and another for pathologic evaluation and immunohistochemistry.

Antibodies and reagents

The monoclonal mouse anti-β-tubulin (1 : 1000) and the polyclonal rabbit antibodies against the long isoform of LEPR (C-terminal) were provided from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-linked anti-mouse/anti-rabbit (1 : 10 000) immunoglobulins were purchased from Amersham Pharmacia (Amersham Pharmacia Biotech, Barcelona, Spain).

Western blotting analysis

The granuloma samples were incubated in 75 μL of lysis buffer containing 20 mmol L^{-1} Tris, pH 8, 1% Nonidet P-40, 137 mmol L^{-1} NaCl, 1 mmol L^{-1} MgCl2, 1 mmol L^{-1} CaCl2, 1 mmol L^{-1} dithiothreitol (DTT), 10% glycerol, 1 mmol L^{-1} phenylmethylsulfonl fluoride and 0.4 mmol L^{-1} sodium orthovanadate, at 4 °C for 30 min on an orbital shaker and then centrifuged at 12 000 g for 15 min. The supernatants were transferred to new tubes. Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as standard. SDS-stop buffer containing 100 mmol L^{-1} of DTT was added to the
periapical tissue samples followed by boiling for 5 min. Fifty microgram protein were loaded in each lane. The samples were then resolved by 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (Martín-Romero et al. 2000). The membranes were blocked with buffered saline–0.05% Tween 20 (PBST) containing 3% bovine serum albumin during 1 h at 23 °C. The blots were then incubated with primary antibody for 1 h, washed in PBST and further incubated with secondary antibodies using horseradish peroxidase-linked anti-mouse/anti-rabbit immunoglobulins. Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (Super Signal from Pierce; Sánchez-Margalet et al. 2003). The bands obtained in the blots were scanned and analysed by the PCBAS 2.0 program (Raytest, Straubenhardt, Germany). The relative optical density of the different bands was normalized by the corresponding intensity of the tubulin immunoblot in each individual experiment.

RNA extraction and quantitative real-time PCR (qRT-PCR) assay and agarose gel electrophoresis

Abundance of LEPR mRNA was determined by quantitative real-time PCR (qRT-PCR). Total RNA was extracted from periapical tissue samples using TRI-SURE reagent (Chomczynski 1993). Concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm. For cDNA synthesis, 5 μg of total RNA was reverse transcribed at 55 °C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche, Mannheim, Germany). Quantitative real-time PCR was performed using the primers based on the sequences of the National Center for Biotechnology Information GenBank database (Table 1). Quantitative RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR Green), and PCRs were performed on a MiniOpticon (Bio-Rad, Hercules, CA, USA). A typical reaction contained 10 μmol L⁻¹ of forward and reverse primer, 3 μL of cDNA, and the final reaction volume was 25 μL. The reaction was initiated by preheating at 50 °C for 2 min, followed by heating at 95 °C for 10 min. Subsequently, 40 amplification cycles were carried out as follows: denaturation 15 s at 95 °C and 1 min annealing and extension at 58 °C. The threshold cycle (CT) from each well was determined by the Opticon Monitor 3 Program (BioRad Laboratories Inc., Richmond, CA, USA). Relative quantification was calculated using the 2⁻ΔΔCT method. For the processed samples, evaluation of 2⁻ΔΔCT indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophilin). The retro-transcribed DNA samples were then resolved by 1% agarose gel; 20 μL samples were loaded in each lane. After running the electrophoresis, gels were visualized directly upon illumination with UV light.

Immunohistochemistry

The excised periapical lesions were fixed in 10% formalin for at least 24 h and then embedded in paraffin and processed routinely. A series of 5 μm sections from each tissue sample were cut. One section of each series was stained with haematoxylin–eosin (H&E) to study the histology and to confirm the diagnosis of apical granuloma. A second section was used for immunohistochemical staining for expression of LEPR.

Pilot studies were undertaken to establish the optimum dilution of the antibody as well as confirming concentration and application times for the chemicals used during the immunohistochemistry (IHC) procedure. Second and third sections were picked up onto a slide, deparaffined in xylene, rehydrated by sequential immersion in a graded series of alcohols and transferred into water for 10 min. Then, specimens were heat treated in sodium citrate buffer (pH 6.0), for 40 min at 96 °C, to unmask antigens. The sections were cooled and washed in phosphate-buffered saline (PBS, pH 7.2). Specimens were incubated in blocking agent (5% albumin bovine serum in PBS) for 1 h. Endogenous peroxidase activity was quenched by incubating the sections in a solution of 3% H₂O₂ in 10% methanol and in PBS for 15 min. Second sections were incubated with rabbit anti-human LEPR monoclonal primary antibodies, respectively, at 1:100 dilutions overnight at 4 °C. The sections were then washed in PBS (3×, 5 min each) and incubated with their respective secondary antibodies. The manufacturer’s instructions were followed for the sequential incubation and durations for the exposure to the

**Table 1** Sequence of PCR primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
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<tr>
<td>Leptin receptor</td>
<td></td>
</tr>
<tr>
<td>Forward: 5'-ATAGTTCCAGTCACCAAAGTGC-3'</td>
<td></td>
</tr>
<tr>
<td>Reverse: 5'-GTCTCGGAGAATCTGATGTC-3'</td>
<td></td>
</tr>
<tr>
<td>Cyclophilin</td>
<td></td>
</tr>
<tr>
<td>Forward: 5'-CTTCCCCCGATACCTCA-3'</td>
<td></td>
</tr>
<tr>
<td>Reverse: 5'-TCTTGGTGGCTACCTC-3'</td>
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secondary antibodies. After washing with PBS, the sections were incubated with dianminobenzidine substrate kit (Dako, Carpinteria, CA, USA) for 5 min that resulted in a brown-coloured precipitate at the antigen–antibody binding sites and the reaction was stopped in distilled water. After removing the slides from water, all the sections were dehydrated and one drop of aqueous mounting medium (Dako Faramount) was applied, and the sections were cover-slipped.

Finally, the immunohistochemical specimens were examined using a Leica Laborlux S Microscope (Leica Microsystems GmbH, Wetzlar, Germany) with a Nikon DSL2 photo digital system (Nikon Corp, Tokyo, Japan). Each sample was analysed with a double-blind system by two different operators under magnifications up to 100×. A cell was considered as positive when it demonstrated distinct brown surface staining. Six representative sites in each sample were photographed at 1.25, 4, 10, 20, 60 and 100× magnification and captured with a software system (CS3, version 10.0.1; Adobe Photoshop, San Jose, CA, USA).

Data analysis
Experiments were repeated separately at least thrice to assure reproducible results. Results are expressed as mean ± standard deviation (SD) in arbitrary units (AU). Arbitrary units were calculated as normalized band intensity in Western blot analysis. Statistical analysis was performed using the GraphPad Prism computer program (GraphPad Software, San Diego, CA, USA).

Results
To define the pathological features of the periapical lesions, paraffin sections of 20 samples were evaluated histologically using haematoxylin and eosin staining. Periapical lesions examined by light microscopy exhibited a large number of infiltrated inflammatory cells, showing zones with the characteristic of the chronic granulomatous inflammatory process: many small capillaries, fibroblasts, numerous connective tissue fibres and inflammatory infiltrate constituted by a dense accumulation of round cells (plasma cells and small lymphocytes), macrophages, foam cells and occasional polymorphonuclear (PMN) leucocytes (Fig. 1a,b). No epithelial cells were observed in these samples, and the specimens were classified as periapical granulomas. Amongst the inflammatory cells in the periapical granulomas, only macrophages were reactive to LEPR antibodies. Plasma cells, lymphocytes, PMNs, fibroblasts or endothelial cells did not show expression of LEPR (Fig. 1c,d).

Figure 1  Histological and immunohistochemical study. Representative sections from periapical granuloma samples evidencing the inflammatory infiltrate. (a) H&E, 40×. (b) H&E, 100×. (c) In situ leptin receptor (LEPR) expression by immunohistochemical, 40×. (d) In situ LEPR expression by immunohistochemical, 100×. Arrows: plasma cells (→), lymphocytes (←), polymorphonuclear cells (→) and macrophages (←).
All human periapical granuloma samples expressed LEPR at both protein and mRNA levels. Western blot analysis revealed the presence in the samples of a protein with apparent molecular weight of 120 kDa, which corresponds to the estimated molecular weight of LEPR (Fig. 2).

To further validate the expression of leptin and LEPR in human periapical granulomas, a real-time PCR assay was performed to examine the messenger RNA (mRNA) levels of LEPR (LEPR/cyclophilin ratio: 1.11 ± 0.16; Fig. 3, upper panel). The size of the amplified fragments (338 bp for LEPR and 194 bp for cyclophilin) was confirmed by agarose gel electrophoresis (Fig. 3, lower panel).

**Discussion**

This study is the first to demonstrate, both immunohistochemically and at the level of mRNA and protein, the expression of LEPR in human periapical granulomas. The expression of leptin in supernatant fluids of explants cultures of human chronic periapical lesions has been previously demonstrated using the ELISA method (Haghighi et al. 2010). LEPR had also been detected previously in experimental rats periapical lesions (Martinez et al. 2007), but mRNA and protein expression studies and the immunohistochemical demonstration of LEPR expression in human periapical inflammatory tissue had not been previously reported. The result of the present investigation, together with those previous findings, suggests that leptin and LEPR expressing cells could be implicated in the pathophysiology of human periapical lesions.

The presence of leptin has been reported in healthy and inflamed gingival tissues (Johnson & Serio 2001, Ay et al. 2012), in gingival crevicular fluid (Bozkurt et al. 2006, Karthikeyan & Pradeep 2007a,b) and in supernatant fluid from cultures of human pulp fibroblasts derived from extracted healthy molar teeth, its production being regulated by neuropeptides (El Karim et al. 2009). Recently, the expression of leptin in human healthy and experimentally induced inflamed dental pulp has been described (Martín-González et al. 2013b). Likewise, the expression of LEPR mRNA in healthy (LEPR/cyclophilin ratio: 0.15 ± 0.02) and experimentally inflamed human dental pulp (LEPR/cyclophilin ratio: 0.23 ± 0.04; $P < 0.05$) has been previously quantified (Martín-González et al. 2013a). The LEPR mRNA level found in the pulp was low compared with that found in periapical granulomas in the
present work (LEPR/cyclophilin ratio: 1.11 ± 0.16). Taking together these results, it can be suggested that LEPR expression correlated with the grade of tissue inflammation. The model of experimental pulp inflammation used in the previous study (Martín-González et al. 2013a), that is high-speed drilling and mechanical pulp exposure followed by pulp extraction after 10 min, could explain the lower level of LEPR mRNA found, compared with its level in periapical granuloma. However, further studies are needed to show the actual expression of this receptor in acute infectious pulpitis in carious teeth.

The primary aminoacid sequence of leptin shows structural similarities to the long chain helical cytokine family, such as IL-2, IL-12 and growth hormone (Zhang et al. 1994, Madej et al. 1995). Leptin and LEPR share structural and functional similarities with the IL-6 family of cytokines (Madej et al. 1995, Tartaglia et al. 1995), and leptin production is increased during infection and inflammation (Fantuzzi & Faggioni 2000, Sánchez-Margalet et al. 2002) upregulating both phagocytosis and the production of pro-inflammatory cytokines by murine macrophages in the acute-phase response (Loffreda et al. 1998, Kiguchi et al. 2009). Leptin affects both innate and adaptive immunity exerting an effect on T cells, monocytes, neutrophils and endothelial cells (Sánchez-Margalet et al. 2003).

Humans periapical granulomas are characterized by an organization of profuse collagen fibres in diverse directions appearing as irregular dense connective tissue with vascular elements. All inflammatory elements of the connective tissue are commonly observed, with a great variety of cell shapes and different cell nuclear organization (Rodríguez et al. 2011). In the present study, all inflammatory cell types characteristic of human periapical granuloma were evident. However, immunohistochemical examination of periapical inflammatory samples only demonstrated the expression of LEPR in macrophages. These findings are in accordance with the previous detection of LEPR mRNA expression in human monocyte/macrophages (Zarkesh-Esfahani et al. 2001). Taking into account that leptin is a potent macrophage chemoattractant (Gruen et al. 2007), the result of the present investigation suggest that leptin plays a role in macrophage chemotaxis during periapical chronic inflammatory response. Lymphocytes, plasma cells and PMNs present in granuloma samples did not express LEPR. On the contrary, LEPR expression has been described during lymphocyte activation in CD8+ T lymphocytes (Martín-Romero et al. 2000) and B lymphocytes (Papathanassoglou et al. 2006).

On the other hand, it has been showed that leptin regulates bone formation (Cirmanová et al. 2008, Turner et al. 2013). The overall effect of leptin on bone might be bimodal. In an osteoblast model, leptin positively promotes ossification in multiple ways including bone mineralization, remodelling, resorption and osteoblast differentiation (Zhang et al. 2013). Therefore, several studies have assessed the correlation between leptin and periodontal disease. A decrease in serum leptin levels following nonsurgical periodontal treatment has been reported (Shimada et al. 2010, Altay et al. 2013). Moreover, LEPR immunoreactivity has been found in the gingival epithelium (Ay et al. 2012) and LEPR gene has been detected experimentally in periapical lesions in rats (Martínez et al. 2007), but no study has yet described the expression of LEPR mRNA and protein in these tissues.

Apical periodontitis is an acute or chronic inflammatory lesion around the apex of a tooth caused by bacterial infection of the dental pulp. The susceptibility to develop pulp and periapical inflammatory processes has been shown to depend upon genetic factors, being influenced by the cytokine genotype/phenotype. Thus, symptomatic dental abscesses predominate in individuals displaying high-producer IL6 genotype (de Sá et al. 2007), Increased IL-1β production contributes to increased susceptibility to apical periodontitis (Morsani et al. 2011), and interleukin (IL)-1 gene polymorphism is associated to root resorption (Bastos Lages et al. 2009).

Conclusion

The expression of LEPR in human periapical granulomas shown in the present study points to a possible role for leptin in the physiology as well as the pathophysiology of the periapical tissues, probably acting as a modulator of periapical immune and inflammatory responses or/and as a link between these processes and cytokine genotype/phenotype.

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